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Expression and Purification of the Amphipathic Form of Rabbit Cytochrome b_5 in *Escherichia coli*

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1. Introduction

Cytochrome b_5 (cyt b_5) is an electron transfer protein that exists in a membrane-bound form in the endoplasmic reticulum where it is anchored to the membrane via a carboxyl-terminal transmembrane α-helix ***(1–3)***. The membrane-bound form of cyt b_5 provides reducing equivalents for the biosynthesis of a variety of lipids including unsaturated fatty acids, plasmalogens, and cholesterol. In addition, it facilitates the cytochrome P450 catalyzed oxidation of selected substrates ***(2)***. The membrane domain is linked to the amino-terminal catalytic heme-containing domain via an 11 amino acid linker. The mammalian cyts b_5 are typically greater than 90% similar in sequence and may be interchangeable in some systems ***(4)***. Nevertheless, our laboratory uses rabbit cytochrome P450 2B4 and cytochrome P450 reductase and has elected to use the rabbit cyt b_5 so all proteins are from a single species. Cyt b_5 also exists in a soluble form in red blood cells where it functions to maintain hemoglobin in its ferrous oxygen-carrying form ***(5)***.

2. Materials

2.1. Escherichia coli (E. coli) *Strains, Media, and Equipment*

1. The key to the marked and reproducible overexpression of the membrane bound form of cyt b_5 is use of the *E. coli* strain C41, a derivative of *E. coli* DE3 (Avidis SA, Saint Beauzire, FR) ***(6)***. This strain moderates the expression of genes downstream from a T7 promoter, thereby, decreasing the toxicity of the large amount of mRNA generated ***(7)*** (Life Technologies-Gibco BRL) (*see* **Note 1**).

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2. LB Agar, 32 g/L water. Autoclave at 121°C for 15 min. Cool and pour into plates. Add carbenicillin to a final concentration of 100 μg/mL (Life Technologies-Gibco RBL).
3. Luria Bertani (LB) medium: 10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, 1 mL 1N NaOH.
4. Terrific broth (TB) medium: prepare by dissolving 12 g of bacto-tryptone, 24 g bacto-yeast extract and 4 mL glycerol in 900 mL water. Sterilize for 20 min and allow to cool. Immediately before use, add 100 mL of a sterile solution of 0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4. If the TB medium is autoclaved in the presence of phosphate buffer, a precipitate will occur.
5. Sterile filtered stock solution of 100 mg/mL carbenicillin made fresh prior to use.
6. 1 *M* isopropyl-1-thio-β-D galactopyranoside (IPTG) in water. Store at −20°C.
7. 200 mM Δ-aminolevulinic acid (Δ-ALA). Store at −20°C.
8. Equipment includes 2.8-L Fernbach flasks, Beckman JA10 rotor, Beckman J2-21 centrifuge (or equivalent), Vibra Cell sonicator (Sonic Materials) 3 mm and 1 cm diameter probe, spectrophotometer (Cary 1, Cary 300 Bio or equivalent). Innova Incubator Shaker 4430 (New Brunswick Scientific, Edison, NJ) or equivalent shaker and autoclave.

2.2. Reagents, Chromatography Resins, and Equipment for Cyt b_5 Purification

1. A 1 m*M* solution of heme is prepared by adding hemin chloride to a solution of 50% ethanol in water and 0.1*N* NaOH. After the hemin chloride dissolves, filter the solution through a 0.2 μm filter. Store at 4°C.
2. Detergents: 10% (v/v) Tergitol NP-10 (Sigma). Store at 4°C. Solid Na deoxycholate (Fisher Biotech).
3. Sodium hydrosulfite (sodium dithionite, [Sigma]).
4. BCA protein assay (Pierce).
5. Mini-Protease inhibitor tablets (Boehringer Mannheim).
6. All buffers should be filter sterilized using a 0.2 μm filter.
7. Buffer A: 10 m*M* phosphate buffer, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 7.0.
8. Buffer B: 10 m*M* phosphate buffer, 1 m*M* EDTA, pH 7.0, 1% Tergitol NP-10.
9. Buffer C: 20 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0 at 25°C, 0.4% Na deoxycholate. 20 m*M* phosphate buffer can be used instead of Tris-HCl.
10. Buffer D: Buffer C, plus 0.4 *M* NaCl.
11. Buffer E: 20 m*M* phosphate buffer, pH 8.0, 1 m*M* EDTA, 0.4% Na deoxycholate.
12. Buffer F: 50 m*M* Tris-acetate, pH 8.1 at 22°, 1 m*M* EDTA.
13. Resins: DEAE Sepharose Fast Flow (Sigma), Superdex-75 prep grade (Amersham Pharmacia Biotech), Sephadex G-25 optional (Sigma).
14. Equipment includes chromatography columns from Bio-Rad, Foxy Jr., or Foxy 200 fraction collector (Isco, Lincoln, NE) or equivalent, Cary spectrophotometer.

3. Methods

3.1. Expression of cyt b_5

1. C41 cells were transformed with the plasmid pLW01-$b_5$mem using standard procedures ***(8,9)***. The transformed cells were plated from a 15% glycerol stock solution onto a LB plate containing 100 μg/mL carbenicillin and incubated overnight at 37°C.
2. A single colony was picked and inoculated into a 2.8 L Fernbach flask containing 500 mL of TB medium supplemented with 0.5 m*M* Δ-aminolevulinic acid and 250 μg/mL carbenicillin.
3. The cultures were incubated at 37°C on an Innova Incubator Shaker 4430 (or equivalent) with shaking at 140 rpm.
4. When the OD of the cultures was 0.35 at 600 nm, IPTG was added to a final concentration of 10 μ*M* and the cells were incubated with shaking for an additional 16–20 h.
5. Remove 2 mL of the culture and set aside for determination of cyt b_5 content (*see* later).
6. The remainder of the cell culture was chilled, poured into 500-mL plastic bottles and centrifuged at 11,000*g* for 10 min at 4°C to pellet the whole cells. The cell culture was centrifuged in a JA10 rotor at 8000 rpm for 30 min at 4°C in a Beckman J2-21 centrifuge.
7. Discard the supernatant and resuspend the cells in ≅ 25 mL of cold Buffer A.
8. Repellet the cells under the same conditions. An average of 8.8 g bright pink cell paste is recovered from 500 mL cell culture (*see* **Note 2**).

3.2. Determination of the Amount of Apocyt b_5 in the Cell Culture and Reconstitution of Holocyt b_5 with Heme

1. Because most of the cyt b_5 is expressed as the apoprotein without the heme, it must be reconstituted with heme prior to purification (*see* **Note 3**). Centrifuge the 2 mL aliquot of the cell culture at 10,000*g* for 1 min at room temperature.
2. Discard the supernatant and resuspend the pink cell pellet in 2 mL Buffer B.
3. Sonicate the cells using a Vibra Cell sonicator (Sonic Materials) with two 30 s pulses at 40% power at 50 W. Immerse the cell suspension in a ice/water slush to keep the temperature below 9.5°C. Cool to 4°C between each pulse. Be sure to sonicate vigorously enough to lyse all the cells. The heme cannot penetrate the bacterial cell membrane and will not be able to reconstitute the apocyt b_5 within the cell. Incomplete reconstitution of the cyt b_5 will result in a poor yield.
4. Dilute the sonicated cells 20-fold with Buffer B and record the absorbance spectrum between 350–650 nm.
5. Add 2 μL aliquots of a ≅ 1 m*M* heme solution to the sonicated cells and record the spectrum after each addition. The difference spectrum (final spectrum-initial spectrum) should resemble the spectrum of cyt b_5 as long as the added heme is forming holocyt b_5 ***(7)***. When the difference spectrum caused by addition of the heme begins to resemble that of the heme and not cyt b_5, the apocyt b_5 has been com-

pletely converted to holocyt b_5. An alternative procedure is to titrate the sonicated cells with the 2 μL aliquots of heme and plot the increase in absorbance at 412 nm. When the apocyt b_5 is completely reconstituted, the absorbance increase at 412 nm produced by a 2-μL heme aliquot will decrease, i.e., the slope of the line found by plotting ΔA at 412 vs heme added will decrease. The point at which the change in slope occurs indicates that the apocyt b_5 has been saturated with heme. Once the amount of heme required to reconstitute the apocyt b_5 in a 2-mL sample is known, the amount of heme necessary to reconstitute the apocyt b_5 in the 500 mL cell culture is readily determined. Reconstitute holocyt b_5 with no more than a ≅ 10% molar excess of heme. If too much excess heme is added, it will be difficult to remove and will interfere with quantitation of cyt b_5.

3.3. Measurement of Holocyt b_5

Holocyt b_5 is measured as previously described ***(10)***. Briefly,

1. Place 1 mL of sample into both a reference and sample cuvet and record a baseline in the spectrophotometer.
2. Reduce the cyt b_5 in the sample cell by addition of ≅ 1 mg of solid sodium dithionite.
3. Record a difference spectrum by subtracting the oxidized spectrum from the reduced spectrum and determine the change in absorbance between 426 and 409 nm. Upon reduction, cyt b_5 increases its absorbance at 426 nm and decreases its absorbance at 409 nm. An extinction coefficient of 185 mM^{-1} cm^{-1} for the absorbance change at 426 minus 409 nm was used to calculate the amount of cyt b_5 present. When the protein is pure and other interfering compounds are absent, an extinction coefficient of 117 mM^{-1} cm^{-1} at 413 nm was used to calculate the amount of cyt b_5 ***(11)***.

3.4. Lysis of E. coli and Membrane Isolation

All of the following procedures with the exception of the chromatography on DEAE were performed at 4°C (*see* **Note 4**).

1. Defrost the pink cell pellet and resuspend in ≅ 25 mL Buffer A.
2. Add two Mini-Protease inhibitor tablets and dissolve them in the cell paste (*see* **Note 5**).
3. Sonicate the cells using a 1-cm-diameter probe that is immersed 2 cm into the cell suspension. Sonicate the cells with ≅ 6 pulses of 2-min duration at 80% power using a 50-W setting. Immerse the cell paste in an ice/water slush during the sonication and do not let the temperature rise above 9.5°C. Other equivalent methods of cell lysis can be used. Regardless of which method of cell lysis is used, it is important to ensure that all cells have been lysed in order to obtain a good yield.
4. Following cell lysis, reconstitute the apocyt b_5 by adding a 10% molar excess of heme based on the amount of apocyt b_5 present in 2 mL of the lysed cell suspension.
5. Centrifuge the sonicated cells at 3000*g* for 15 min at 4°C to remove any unlysed cells. If the pellet is red, resonicate to lyse any remaining intact cells.
6. Centrifuge the membrane containing supernatant at 100,000*g* for 1 h at 4°C.

7. Discard the supernatant and resuspend the cyt b_5 membrane containing pellet in ≅ 30 mL of Buffer B. The pellet can be resuspended by using either a teflon homogenizer or a brief 30s sonication pulse.
8. After resuspending the cell pellet, dilute to 100 mL with Buffer B and stir at 4°C while determining the protein concentration of the solution using the BCA assay.
9. Dilute the cyt b_5 containing membranes with Buffer B to a volume which will give a protein concentration of 4 mg/mL. The final volume is usually ≅ 150 mL with a detergent: protein (w/w) ratio of 2.5:1.
10. Stir at 4°C for ≅ 3 h to solubilize the cyt b_5.
11. Centrifuge the solubilized cyt b_5 at 100,000*g* for 1 h. The pellet should be almost colorless.
12. Load the dark red supernatant which contains the cyt b_5 onto the DEAE-Sepharose column.

3.5. DEAE-Sepharose Chromatography

1. Equilibrate a 2.5 × 16-cm column of DEAE-Sepharose with ≅ 1 L of Buffer C (*see* **Note 6**). The DEAE-Sepharose chromatography was performed at room temperature in order to prevent deoxycholate gel formation which occurs at 4°C, pH less than 8.0, and high-salt concentration.
2. Load the cyt b_5 containing solution onto the column at a rate of ≅ 3 mL/min. Cyt b_5 will bind to the top one-third of the column.
3. Wash the column with ≅ 300 mL of Buffer C.
4. Elute the cyt b_5 with a linear gradient formed with equal amounts of Buffer C and Buffer D (i.e., Buffer C in 0.4 *M* NaCl).
5. Pool the fractions with an A_{412}/A_{280} ratio greater than 1.6 and then concentrate in a 50-mL Amicon stirred cell using a YM-10 membrane. An equivalent method of concentration such as Centriprep can be used.
6. When the volume has been reduced to ≅ 30 mL, dilute four-fold with 120 mL of Buffer C and reconcentrate to ≅ 30 mL to decrease the salt which can cause gelling of the deoxycholate containing buffer. The sample is now ready to be applied to the sizing column.

3.5. Superdex-75 Chromatography

1. Prepare a 5 × 62-cm column with Superdex-75 prep grade and equilibrate with Buffer E. At a flow rate of 0.25 mL/min it can be equilibrated over the weekend.
2. Apply the concentrated sample carefully to the column, taking care not to disturb the top of the column. The smaller the volume loaded onto the column the better the resolution. Load the sample by gravity flow at a rate of ≅ 0.5 mL/min.
3. Begin eluting the column by gravity flow with Buffer E until the cyt b_5 has entered 4 cm of the column. The rosey pink protein should elute in a regular band for best resolution.
4. At this point, add a pump to elute the column slowly at a rate of 1 mL/min. In 0.4% deoxycholate, cyt b_5 elutes as a dimer of 35 kDa ***(3)***.
5. Combine fractions with an A_{412}/A_{280} nm ratio greater than 2.5.

6. The deoxycholate can be removed either by extensive dialysis against 20 m*M* KPO_4 buffer pH 8.0 and 1 m*M* EDTA or size-exclusion chromatography on a Sephadex G25 column (1 × 100 cm) preequilibrated with 10 m*M* KPO_4 buffer pH 8.0 and 1 m*M* EDTA.

This procedure should yield approx 120 mg of pure protein from 500 mL of *E. coli* cell culture.

4. Notes

1. Contact information for obtaining C41 from Avidis: isabelemounier@avidis.fr, or fergalhill@avidis.fr, Tel: +33(0)4 7364 4390, Fax +33 (0)4 7364 4393.
2. In previous procedures, our laboratory and other laboratories have reported significant proteolysis of the membrane-bound form of cyt b_5 to the soluble form of cyt b_5 during bacterial cell lysis ***(12,13)***. The simple procedure of washing the cells in buffer removes a significant amount of the proteases found in the cell culture medium and other contaminating proteins. The marked decrease in proteases during cell lysis is one of the factors that allows complete recovery of the membrane bound form of cyt b_5. No soluble cyt b_5 is formed during the purification procedure if appropriate precautions are taken.
3. Only approx 10% of the cyt b_5 expressed under our experimental conditions is holo protein. The remaining 90% is apoprotein which must be reconstituted with heme as soon as possible after cell lysis because the apo form is more susceptible to proteolysis.
4. Because loss of the membrane anchor of cyt b_5 by proteolysis results in inactivation, the purification procedure is performed under "almost" sterile conditions with filtered buffers (0.2-μm filters) to prevent contamination.
5. The protease inhibitors must be present during cell lysis to prevent cleavage of the hydrophobic membrane anchor from the amphipathic form of cyt b_5.
6. Equilibration of the DEAE column is critical. The column is adequately equilibrated when the pH and resistance of the buffer eluting from the column is identical to that being loaded onto the column. Proper equilibration ensures good resolution and reproducibility with the DEAE-Sepharose column.

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Dihydroorotate Dehydrogenase of *Escherichia coli*

Kaj Frank Jensen and Sine Larsen

1. Introduction

1.1. Different Types of Dihydroorotate Dehydrogenases (DHODs)

Dihydroorotate dehydrogenase (DHOD) catalyzes the fourth reaction in the pathway for *de novo* synthesis of UMP and forms the 5,6-double bond of the pyrimidine base. In this reaction, two electrons and two protons are transferred from dihydroorotate to an electron acceptor that varies between different types of the enzyme. Sequence alignments have shown that all DHODs contain a polypeptide chain that is encoded by a *pyrD* gene. This polypeptide forms the catalytic core structure, folding into an $(\alpha/\beta)_8$-barrel. The active site, which contains a tightly bound molecule of flavin mononucleotide (FMN), is formed by loops that protrude from the top of the barrel (e.g., **ref. *1***). The first half reaction, in which the enzyme is reduced and dihydroorotate is oxidized to orotate, is initiated by binding of dihydroorotate at the *si*-side of the isoalloxazine ring of FMN ***(2)*** and, after abstraction of a proton from the 5'-position of dihydroorotate by a cysteine or a serine residue in the enzyme, a hydride ion is transferred to FMN from the 6-position of the substrate ***(3,4)***. The first half reaction is common to all DHODs, but different types of DHODs deviate from each other in quaternary structure, subcellular location, and use of electron acceptors to reoxidize the reduced enzyme in a second half reaction ***(5)***.

1.1.1. The Soluble Class 1 DHODs

The class 1 DHODs are soluble proteins. Two types have been identified. Class 1A DHODs are dimeric proteins able to use fumarate as electron acceptors. The enzymes are found in milk fermenting bacteria like *Lactococcus lac-*

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tis ***(6,7)*** and *Enterococcus faecalis* ***(8)***, in the anaerobic yeast *Saccharomyces cerevisiae* ***(9,10)*** and in some eukaryotic parasites ***(11,12)***. The enzyme from *L. lactis* (DHODA) has been studied in considerable detail and the crystal structure has been solved of the free enzyme and as a complex with the product orotate ***(1,2)***.

Class 1B DHODs are heterotetrameric enzymes that use NAD^+ as electron acceptor ***(13)***. The occurrence is restricted to Gram positive bacteria. The closely related strains *L. lactis* ***(14)*** and *E. faecalis* ***(15)*** have both a class 1A and a class 1B DHOD ***(6)***, but species of *Bacillus* ***(16,17)*** and *Clostridium* ***(4,18)*** only possess a class 1B enzyme. The protein from *L. lactis* (DHODB) has been studied in detail ***(13)*** and the crystal structure has been solved for the free enzyme and as a complex with the product orotate ***(19)***. Two of the subunits are encoded by the *pyrDb* gene, and together they form a dimeric protein like DHODA. Associated with this catalytic core are two tightly bound electron transfer subunits, which are encoded by the *pyrK* gene and protrude from the catalytic dimer like two moose horns. The PyrK polypeptides belong to the ferredoxin reductase superfamily. They have flavin adenine dinucleotide (FAD) and a [2Fe-2S] cluster as cofactors and are engaged in the channeling of electrons to NAD^+ ***(13,19)***.

Other types of soluble DHODs exist. For instance, a class 1B-like DHOD able to use molecular oxygen, but not NAD^+, has been found in *Lactobacillus* and is devoid of an electron transfer subunit ***(20,21)***. In addition, the archaeon *Sulfolobus solfataricus* has a class 1B-type DHOD associated with an iron-sulfur cluster protein different from PyrK. The electron acceptor preferences of this protein is unknown ***(22)***.

1.1.2. The Membrane Associated Class 2 DHODs

The membrane associated class 2 DHODs use quinones of the respiratory chain as electron acceptors. They are found in Gram negative bacteria like *E. coli* ***(23)*** and *Helicobacter pylori* ***(24)***, where they are associated with the cytoplasmic membrane, and in most eukaryotic organisms, where are anchored in the inner mitochondria membrane ***(25)***. The class 2 enzymes are monomeric proteins with a strong tendency to aggregate ***(26,27)***. The core part of the enzymes, with the active site, forms an $(\alpha/\beta)_8$-barrel structure similar to the structure of the class 1 enzymes ***(28,29)*** although the sequence similarity between the two classes of DHODs is very low, 12–20% identity ***(5,30)***. The polypeptide chains of all class 2 enzymes are extended in the N-terminal relative to the class 1 enzymes (*see* **Fig. 1**). In bacteria this extension sequence is just a little more than 40 amino acid residues. In the *E. coli* enzyme (DHODC) it forms a separate helical domain with a hydrophobic cavity between two of the helices, located at the side of the core domain ***(28)***. The small N-terminal

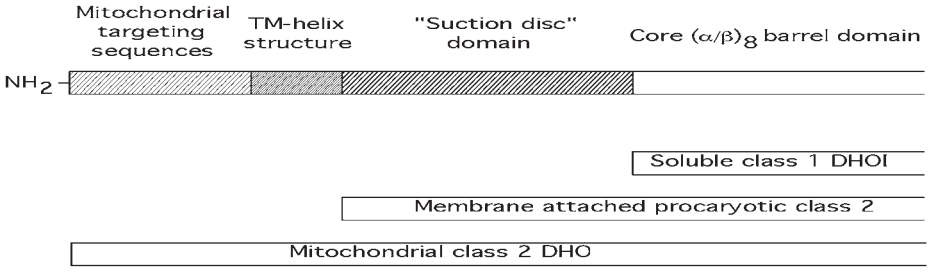

Fig. 1. Schematic representation of the functional roles of sequence elements in the polypeptide chains of different dihydroorotate dehydrogenases. *See* main text for further explanation.

domain enables DHODC to use respiratory quinones as electron acceptors (menaquinone appears to be the physiological electron acceptor of DHODC ***[31]***) and is essential for the association of the enzyme to the membrane by a mechanism that essentially is unknown ***(28)***. We call the domain "a suction disk", but do not know if the quinones, which bind to this domain, are involved in membrane association through their long hydrophobic tails.

The mitochondrial class 2 DHODs share the "suction disk domain" with the enzymes of prokaryotic origin ***(29)***, but the N-terminal extensions of the mitochondrial enzymes are longer than their prokaryotic counterparts, as they contain a short segment of 16–20 amino acid residues which (from the sequence) is predicted to form a transmembrane helix just upstream of the "suction disk structure" and carry sequences that target the proteins for import in mitochondria ***(25)*** (*see* **Fig. 1**).

Our current procedure for overexpression, purification, and crystallization dihydroorotate dehydrogenase from *E. coli* consists of the following major steps:

a. Growth of cells and over-production of DHOD from a plasmid encoded, inducible gene.
b. Disruption of cells by ultrasonic treatment and release of the enzyme from membranes by Triton X-100 in the crude extracts.
c. Chromatography on a DE-52 anion exchange column in the presence of Triton X-100.
d. Hydrophobic interaction chromatography on a column of Phenyl Sepharose and elution with Triton X-100.
e. Chromatography on a second anion exchange column to remove the detergent.
f. Crystallization with sodium formate as precipitant.

The procedure yields about 20 mg DHODC per liter bacterial culture ***(27)***. The crystal structure was published by Nørager et al. ***(28)***.

2. Materials

1. *The expression plasmid:* The expression vector pAG1 ***(27)*** is a derivative of the ampicillin resistance plasmid pUHE23-2 ***(32)***. It carries the 336 codons reading frame of the *E. coli pyrD* gene, encoding DHODC, cloned behind the strong $T7_{A1/04/03}$ promoter, which is a synthetic derivative of the $T7_{A1}$ early promoter and contains two operator sites for binding the LacI repressor.
2. *Bacterial strains:* The *E. coli* strain SØ6645 (*araD139*Δ*(ara-leu)7679 galU galK*Δ*(lac)174*Δ*pyrD(BssHII-MluI::Km*^{r}) [F' *proAB lacI*^{q}*Z*Δ*M15Tn10*] overproduces the LacI repressor from the *lacI*^{q} gene on the episome and is deleted for the promoter proximal part of the chromosomal *pyrD* gene ***(7)***. Strain SØ6735 (*rph-1 metA recA56 srl*::Tn*10*) [F' *proAB lacI*^{q1}*Z*::Tn*5*] ***(28)*** is derivative of the methionine requiring strain DL41 previously used for production of selenomethionine substituted proteins ***(33)***.
3. Preswollen diethyl aminoethyl cellulose (DE-52) is available from Whatman Ltd. (Maidstone, England).
4. Phenyl Sepharose® CL-4B is available from Pharmacia LKB (Uppsala, Sweden).
5. LB-broth: 10 g Bacto® Tryptone (Difco, Detroit, MI), 5 g yeast extract (Oxoid Ltd., Basington, UK) and 5 g NaCl per liter of ion exchanged water. If needed, the pH was adjusted to 7.0 by addition of NaOH before autoclaving ***(34)***.
6. Solution A: 20 g $(NH_4)_2SO_4$, 75 g $Na_2HPO_4.2H_2O$, 30 g KH_2PO_4, and 30 g NaCl per liter.
7. Solution B: 20 mL of 1 *M* $MgCl_2.6H_2O$, 2 mL of 0.5 *M* $CaCl_2.2H_2O$, and 3 mL of 10 m*M* $FeCl_3.6H_2O$ per 9 L ***(35)***.
8. (A +B) basal salt medium: Mix together autoclaved Solution A and Solution B, one part A to nine parts B.
9. Supplements are added from sterile solutions that had been autoclaved separately at 100°C, glucose as a 20% solution, amino acids at a concentration of 5 mg/mL and uracil 2 mg/mL. Ampicillin (Sigma, St. Louis, MI) and isopropyl-β-D-thiogalactoside (IPTG; Bingswood Industrial Estate, Whaley Bridge, UK) are added as solid material.
10. Buffer A: 5 m*M* sodium phosphate pH 7.0 containing 0.25 m*M* ethylenediamine tetraacetic acid (EDTA) (*see* **Note 1**).
11. Buffer B: 5 m*M* sodium phosphate pH 7.0, 0.25 m*M* EDTA, 5 m*M* $MgCl_2$, 0.1% Triton X-100 (Sigma).
12. Buffer C: 50 m*M* sodium phosphate, pH 6.2, containing 0.1 m*M* EDTA, and 0.1% Triton X-100.
13. Buffer D: 50 m*M* sodium phosphate pH 7.0 containing 0.1 m*M* EDTA.
14. Centrifugation spin columns (Amicon, Centriprep®).

3. Methods

3.1. Growth and Harvest of Cells

Strain SØ6645 transformed with the expression plasmid pAG1 was grown at 37°C in LB-broth medium containing ampicillin (100 mg/L). To ensure a high production of DHODC we always use freshly transformed cells (*see* **Note 2**). A preculture (100 mL) is inoculated in the morning with 4–5 single colonies from

a fresh transformation agar plate and grown to an OD_{436} of circa 0.5, when it is cooled in an ice bath. The preculture is stored at 4°C overnight and diluted into 2 L of prewarmed medium on the morning of the next day (*see* **Note 3**).

The culture is grown with vigorous aeration by shaking. IPTG (0.5 m*M*) is added at OD_{436} = 0.7–1.0 to induce expression of the *pyrD* gene and growth is continued overnight, while the culture reaches stationary phase at an OD436 about 5 (*see* **Note 4**). Cells are harvested by centrifugation, washed with 0.9% sodium chloride and frozen at –20°C. The cell-pellet is strongly yellow because of the content of FMN in DHODC.

3.2. Extraction and Purification

All operations during purification are carried out at 4°C.

3.2.1. Extraction

1. Frozen cells from 2 L culture (circa 16 g) are resuspended in 80 mL of buffer A and disrupted by ultrasonic treatment.
2. Add $MgCl_2$ to a final concentration of 5 m*M,* and Triton X-100 to a final concentration of 0.1% to dissolve the membranes.
3. The extract is cleared by centrifugation in an SS-34 rotor (Sorvall) at 13,000 rpm (20,000*g*) for 1 h (*see* **Note 5**).

3.2.2. First Chromatography on DE-52

1. The clear yellow extract is pumped (flow 1 mL/min) onto a column of DE-52 (1.6 × 25 cm) equilibrated with buffer B. The enzyme binds in a narrow zone at the top of the column.
2. After application of the sample, the column is washed first with 50 mL of buffer B and then with 50 mL of buffer C.
3. The enzyme is eluted with a linear gradient (400 mL) from 0 to 0.25 *M* sodium chloride in buffer C, while 10 mL fractions are collected. The enzyme appears from the column with a peak around 0.15 *M* NaCl.

3.2.3. Hydrophobic Interaction Column Chromatography

1. The active, yellow fractions from the DE-52 column are pooled and solid ammonium sulfate is dissolved in the liquid at a final concentration of 1.1 *M*.
2. A turbidity that forms after the addition of ammonium sulfate is removed by centrifugation (10 min at 12,000*g*). The pellet is colorless.
3. The clear supernatant is pumped (flow 0.5 mL/min) onto a column of Phenyl Sepharose (1.6 × 20 cm) which has been equilibrated with buffer D containing 1.1 *M* ammonium sulfate. The enzyme binds in a highly concentrated zone at the top of the column.
4. After application of the sample, the column is washed with a linear gradient (160 mL) from 1.1 *M* to 0 *M* ammonium sulfate in buffer D followed by 100 mL of

buffer D. The washing removes a substantial amount of contaminating protein, but DHODC remains bound although it spreads a little on the column during the wash.
5. The enzyme is eluted as a sharp peak by pumping buffer D containing 1% Triton X-100 through the column. This high concentration Triton X-100 gradually replaces the enzyme from the column, and the column material changes appearance to a more white and nontransparent texture above moving yellow zone of DHODC (*see* **Note 6**).

3.2.4 Second DE-52 Column Chromatography

1. The pooled fractions from the Phenyl-Sepharose column are loaded on a second DE-52 column (1.6 × 25 mL) equilibrated with buffer D. The flow rate is 1 mL/min.
2. The column is washed thoroughly with about 300 mL of buffer D to remove all Triton X-100, which is monitored by the UV-light absorption at 280 nm.
3. The enzyme is eluted in a somewhat broad peak by a linear gradient (400 mL) from 0 to 0.3 *M* sodium chloride in buffer D. The chromatography on the DE-52 column in the absence of Triton X-100 results in a loss of about one-third of DHODC, which remains stuck at the top of the column even at very high concentrations of NaCl, but we have accepted this loss of enzyme in order to be able to replace Triton X-100 with other detergents (*see* **Note 7**).

3.2.5. Concentration and Storage

1. The active fractions from the second DE-52 column are pooled and concentrated using centrifugation spin columns (Amicon, Centriprep®).
2. For most purposes, the enzyme was dialyzed against buffer D containing 50% glycerol and stored in liquid form at −20°C at a concentration around 10 mg/mL.
3. Prior to crystallization the protein sample was dialyzed against a solution of 25 m*M* of sodium phosphate pH 7.0 containing 0.1 m*M* EDTA and 10% glycerol and stored in 0.5 mL aliquots at −20°C.

3.3. Crystallization

The crystallization of DHODC has been described previously by Rowland et al. ***(36)***. Crystals were obtained by the vapor diffusion technique using 5 μL sitting drops in microbridges placed over a 0.6-mL reservoir solution in the Linbro plates closed with cover slides. The experiments were carried out at room temperature. The drops were made from 2.5 μL protein solution (12–15 mg/mL DHODC) and 2.5 μL of the reservoir solution. Crystals could be obtained with reservoir solutions that have the following composition: 0.1 *M* sodium acetate, sodium formate in the concentration range 3.9–4.4 *M*, pH 4.0–5.5, and 25 m*M* β-n-octyl β-D-glucoside (β-OG). Prior to equilibration the drops had a composition contained 6.0–7.5 mg/mL of DHODC, 12.5 m*M* sodium phosphate pH 7, 0.05 m*M* EDTA, 5% glycerol (from the protein solution), 12.5 m*M* β-OG, 0.05 *M* sodium acetate, and 1.95–2.2 *M* sodium formate with a pH of 4.0–5.5, while

the reservoir solutions contained 0.1 *M* sodium acetate, and 3.9–4.4 *M* sodium formate with a pH of 4.0–5.5. With reservoir solutions in the afore mentioned range of sodium formate concentrations and pH, the enzyme crystallized within 1–2 wk as yellow needles of the approximate dimensions $1.5 \times 0.15 \times 0.15$ mm. The crystals have small whiskers at one end that was cut away to make the crystals suitable for X-ray diffraction experiments. To be able to measure diffraction data from crystals under cryogenic conditions, the crystals had to be soaked for a few seconds in a cryoprotecting reagent containing 4.5 *M* sodium formate, 0.1 *M* sodium acetate at the crystallization pH and 10% glycerol. The X-ray diffraction experiments showed that the crystals are tetragonal. To overcome the phase problem the selenomethionine substituted protein was prepared (*see* **Note 8**). It could be crystallized under the same conditions as the native enzyme. The structure determination was achieved by the MAD (multiple anomalous dispersion) method based on diffraction data collected with synchroton radiation at three different wavelength around the Se-absorption edge. Further details are described by Nørager et al. ***(28)***.

4. Notes

1. Buffers are prepared using doubly distilled water. They were prepared by dilution of five-times concentrated stock solutions and mixed with NaCl from a 5 *M* NaCl stock solution that was passed through a nitrocellulose filter to remove unwanted particles.
2. Other *E. coli* strains can be used, but the F' *proAB lacI^{q}ZΔM15 Tn10* episome, which directs the overproduction of the LacI repressor, is needed because the plasmid does not itself carry a *lacI* gene. The overproduction of all types of DHOD is toxic to *E. coli* and transformation with plasmid pAG1 is not possible unless the DHOD expression is kept repressed.
3. It is advisable to use freshly transformed cells and keep the culture exponentially growing until the final culture reaches stationary phase before harvest. If growth of culture is interrupted, it should preferably be done at a low cell density, e.g., at $OD_{436} \leq 0.5$. If the preculture has been grown into stationary phase, plasmid-free cells tend to outgrow the plasmid containing cells when the preculture is diluted into fresh medium, because the added ampicillin is rapidly broken down. This behavior is in all likelihood related to the fact that the copy number of plasmid pAG1 (and other relaxed plasmids), and hence the production of β-lactamase, increases dramatically when the culture approaches stationary phase. It is possible to store the transformed cells if an aliquot of the uninduced culture at a low cell density ($OD_{436} \leq 0.5$) is mixed with 20% glycerol and the frozen at –20°C. However, in that case, it is advisable to spread the cells to single colonies on an LB-agar plate with 0.1 mg/mL of ampicillin and test a few colonies for high-protein production in small cultures.
4. An "autoinduction" of *pyrD* expression from plasmid pAG1 occurs at a cell density about $OD_{436} = 2$. The reason is that the concentration of repressor binding sites on pAG1 in cultures approaching stationary phase exceeds the amount of LacI repressor produced from the stringently controlled F'-episome. The production of

DHODC from pAG1 is almost as high in "uninduced" stationary cultures as it is in cultures that are induced by addition of IPTG, but because this "autoinduction" may depend on subtle differences in the culture conditions, we have retained the induction with IPTG as described. The "autoinduction" of protein expression in stationary cultures, which we have seen with several plasmids where repression relies upon a *lacI* gene on an F'-episome, may also contribute to the strong tendency of plasmid-loss and low protein production in cultures that are inoculated with outgrown precultures.

5. When DHODC was purified from bacteria that expressed the protein either from the chromosomal *pyrD* gene or from low production plasmids ***(23)*** we disrupted the cells by use of a French press and isolated the membranes, which contained near 100% of the enzyme, by centrifugation. The protein was then released from the membranes by addition of Triton X-100 ***(37)***. The isolation of membranes prior to release of the enzyme gave a substantial purification (≥ 10-fold), but with the large overproduction of DHODC, achieved by the use of plasmid pAG1, the majority of DHODC remains in the supernatant, when the membranes are isolated. Therefore, this step is omitted from the purification procedure and the membranes are dissolved by addition of Triton X-100 prior to all fractionation.
6. The Phenyl-Sepharose column can be regenerated by extensive washing with 20% ethanol in water. The removal of Triton X-100 can be followed by monitoring the UV-absorbance.
7. The behavior of DHODC during chromatography on the DE-52 ion column in the absence of detergent is unusual. At low ionic strength, the enzyme appears to bind to the column material primarily by electrostatic forces and be released by a moderate salt concentrations. However, at high-salt concentrations, it sticks to the column material by hydrophobic interactions. In an attempt to elute the protein from the DE-52 column in a more-concentrated manner than obtained by the described salt gradient, we applied a solution of 1 *M* NaCl in buffer D to the column directly after Triton X-100 had been removed. All of the enzyme remained at the column during the high salt wash, and a part of it (about two-thirds) was eluted by a backward gradient from 1 *M* to 0 *M* NaCl in buffer D with a peak about 0.15 *M* NaCl.
8. Strain SØ6735 transformed with pAG1 was used to produce selenomethionine substituted DHODC for crystallization and structure determination ***(28)***. The strain was grown in the phosphate buffered minimal (A+B)-medium ***(35)*** supplemented with glucose (0.5%), methionine, leucine, isoleucine, and valine (all at a concentration of 50 mg/L) and with uracil (20 mg/L) and ampicillin (100 mg/L). Uracil was added because the *rph-1* mutation in strain DL41 (a derivative of MG1655) has a polar effect on transcription of the *pyrE* gene, which generates a strong stress in the supply of pyrimidine nucleotides and a reduced growth rate in pyrimidine free media ***(38)***. The preculture was grown in a medium supplied with normal L-methionine. At OD_{436} = 0.5 the preculture was cooled in an ice bath. The cells were harvested by centrifugation, washed with basal salt medium, and resuspended at an OD_{436} = 0.05 in 2 L of prewarmed medium, similar to the medium described aforementioned, but with DL-selenomethionine (0.1 g/L) replacing L-methionine.

After a few minutes, the growth rate declined to half of that seen in the preculture, indicating that all L-methionine had been consumed and that the cells were now thriving on selenomethionine. The synthesis of DHODC was induced at OD_{436} = 0.5 and the culture was left to reach stationary phase overnight at an OD_{436} of 2–3. Harvest of the cells, extraction, and protein purification was performed as aforementioned with the notable exception that 1 m*M* dithiothreitol (DTT) was included in all the buffers to prevent oxidation. Furthermore, only 0.9 *M* ammonium sulfate was added to the enzyme solution prior to application on the Phenyl-Sepharose column and the subsequent gradient changed accordingly to go from 0.9 *M* to 0 *M* ammonium sulfate. The reduction in the ammonium sulfate concentration was made because the selenomethionine substituted DHODC precipitates in the presence of 1.1 *M* ammonium sulfate. The yield of DHODC, fully substituted with selenomethionine, was circa 10 mg per liter of medium was.

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3

A General Approach for Heterologous Membrane Protein Expression in *Escherichia coli*

The Uncoupling Protein, UCP1, as an Example

Alison Z. Shaw and Bruno Miroux

1. Introduction

It is well accepted that one of the major limitations in membrane protein structure determination is to obtain enough protein material (for review, *see* **ref. *1***). A batch of approx 5 mg is necessary for the first round of crystallization screening, for example. Because many of the most interesting membrane proteins are often expressed at very low levels, it can be difficult, and perhaps not desirable, to purify them from their natural source; using an overexpression system is the obvious alternative. Owing to the great range of expression vectors available and the ease of use, *Escherichia coli* has proven to be the expression host of choice, particularly for small, cytoplasmic proteins. Heterologously expressed membrane proteins are often toxic to *E. coli*, which prevents cell growth and limits protein yields. In this chapter, we describe how an "in vitro evolution" approach can be used to produce *E. coli* strains, e.g., C41(DE3) and C43(DE3), which are better suited than BL21(DE3) to expression of some membrane proteins. Expression of the uncoupling protein (UCP1) in C41(DE3) is given as an example.

UCP1 plays a part in what is known as "proton leak." This curious phenomenon has been recognized for many years and is defined as the dissipation of the proton gradient existing across the inner mitochondrial membrane by routes other than through ATP synthase. The significance of this phenomenon is gradually coming to light through the study of the uncoupling protein family. UCP1, the most well-characterized member is known to provide this "unproductive" proton conductance pathway in brown adipose tissue (BAT) mitochondria and

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is activated during nonshivering thermogenesis, leading to the production of heat (for review, *see* **ref. *2***). The recent cloning of UCP2 and UCP3, both exhibiting high sequence identity with UCP1 has promoted interest in this field ***(3,4)***. They are expressed in tissues other than brown adipose tissue and provide potential therapeutic targets for the treatment of metabolic diseases such as diabetes and obesity and of inflammatory diseases ***(5–7)***.

Hydropathy analysis of these 33-kDa membrane proteins proposes six transmembrane domains, confirmed for UCP1 by antibody mapping which also reveals the location of N and C terminals in the intermembrane space ***(8)***. The organization of UCP1 into three repeats of approx 100 amino acids is apparent from the amino acid sequence, with each domain containing two transmembrane segments ***(9)***.

Both the full-length rat UCP1 and fragments of the protein (corresponding to each of the three repeats) have been expressed with histidine tags, in *E. coli* C41 (DE3) ***(10)*** as inclusion bodies. A method for the expression is described here, alongside a protocol to generate improved *E. coli* strains for membrane protein expression and some tips on finding the best conditions for maximizing protein yields.

2. Materials

1. 1X LB Medium: 10 g Bacto Tryptone (Difco #0123-17-3), 5 g Bacto Yeast Extract (Difco #0127-17-9), 5 g NaCl. Add sterile water to 1 L. Adjust the pH to 7.2–7.5 with NaOH (a few pellets of the solid) and autoclave.
2. 2X TY Medium: 16 g Bacto Tryptone (Difco #0123-17-3), 10 g Bacto Yeast Extract (Difco #0127-17-9), 5 g NaCl, 2 g glucose. Add sterile water to 1 L. Adjust the pH to 7.0 with NaOH (a few pellets of the solid) and autoclave.
3. IPTG (Isopropyl-beta-D-galactoside): A 1 M stock can be prepared in water and stored at −20°C. IPTG can be obtained from Sigma-Aldrich.
4. Antibiotics: Working concentrations of some antibiotics:

Antibiotic	(μg/mL)
ampicillin	100
carbenicillin	100
chloramphenicol	34
kanamycin	30
rifampicin	200
tetracycline	12.5

5. 5X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 4.0 mL distilled water, 1.0 mL 0.5 M Tris-HCl, 0.8 mL glycerol, 1.6 mL10% SDS, 0.4 mL β-mercaptoethanol, 0.2 mL 0.05% (w/v) bromophenol blue.
6. Inclusion body buffer: 300 m*M* NaCl, 10 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.5, 4 m*M* phenyl methyl sufonyl fluoride, (PMSF) (Sigma-Aldrich), added at the time of use. A 1 *M* stock of PMSF can be prepared in methanol and stored at −20°C. This protease inhibitor is highly toxic.

3. Methods

3.1. Designing Contructs for Expression

As well as making expression constructs of the full-length protein, it may make sense to break up your protein into smaller fragments. In that case, it is essential to think carefully about where to put the boundaries. Check hydropathy data and use programmes such as SMART (protein domain identification) and Jpred (secondary structure prediction) to identify potential domain boundaries (*see* **Note 1**). In general, it is best to avoid having hydrophobic residues on the ends of protein constructs because they may induce aggregation.

3.2. Choosing Expression Vectors

Having decided on which sequences to clone, the next step is to choose from the plethora of expression vectors. It is very difficult to know which vector will give the highest yields for the particular protein of interest and often the best strategy is to try a number of different vectors in parallel and screen for expression on a small scale (*see* **Subheading 3.4.**).

Optimal conditions are achieved when the plasmid remains stable throughout the growth of the culture and when expression of the protein is slighly toxic to the cell, but not so much that cell growth is seriously impaired. Unfortunately, expression systems are unpredictable because the strength of the promoter depends very much on the stability of the mRNA of the target gene ***(11)***. For instance, if the mRNA is very stable then a weak promoter will make a good balance to achieve a high level of expression of the target gene without toxicity to the host. On the other hand, if the mRNA of the target gene is highly unstable, then even the strongest *E. coli* promoter will be useless. Therefore, as a general rule, we advise you to clone your target gene in two to three very different expression vectors and to transform two to three different bacterial strains in order to cover weak, intermediate, and strong expression systems. In the case of the well-known T7 based RNA polymerase expression system ***(12)***, the low copy number pET vectors (Novagene) could be tested alongside the high copy number pRSET vector (Invitrogen) or the pMW7 vector ***(13)***. Other low or high copy number vectors with weak promoters should also be tested, such as the arabinose promoter or the lac promoter (*see* **Note 2**).

3.3. The Bacterial Host

When it comes to expressing the protein, the main difficulties are to balance the efficiency of protein production with the toxicity of expression; and to optimize yields of correctly folded protein without driving the equilibrium too far toward formation of inclusion bodies. This depends very much on the combination of vector and bacterial host. In this section we describe a general method

that allows you to assess (*see* **Subheading 3.3.1.**) and improve your expression system by selection of the bacterial host (*see* **Subheading 3.3.2.**).

3.3.1. Identifying a Suitable Host Strain

The method relies on the fact that the growth behaviour of your host strain on solid medium (LB-agar plates) will closely reflect its behaviour in liquid medium. For instance, if the bacterial host harboring the plasmid is unable to form a colony on an agar plate containing the expression inducer, then in liquid medium, cells may well start to die once expression is induced, and may lose the plasmid very rapidly. Eventually cells lacking the expression vector will overgrow the culture and very little heterologous protein will be obtained. This is what we have observed in experiments using the T7 expression system ***(10)***.

A strategy for identifying a suitable host is described in **Fig. 1**. It can be divided into several stages, the first of which is to check that your empty vector is not toxic to your bacterial host, especially if you use a T7 expression system ***(14)***. Cells harboring vectors that do not contain your inserted gene should form regular sized colonies on plates containing the inducer. If not, then you should either change the combination of vector and host or select mutant hosts by following the procedure described in **Subheading 3.3.2.** (*see* **Fig. 2**).

This first step is important so you can be sure that any potential toxicity comes as a result of the inserted gene and is not from the plasmid alone. You should now proceed with the vector containing the membrane protein gene and check the appearance of colonies on both LB-Agar plates (+antibiotic) and on plates also containing inducer. It is possible that only very small colonies form on the LB-agar plates, even without inducer (*see* **Note 3**). The only option in that case is to try a new host or a new vector.

Provided the colonies are normal sized in the absence of inducer, you should see how they look when inducer is added to the plates. As shown in **Fig. 1**, there are three possibilities at this stage. The first possible outcome is that the size of the colonies does not change at all with or without the inducer. This is a bad sign and probably indicates that the level of expression of your protein is very low or at least not optimal. Of course you can proceed and check the expression level of the target gene in liquid medium, but it is also wise to change to a stronger expression system.

The second possibility is that cells form smaller colonies in the presence of the inducer. This is a good sign but does not always guarantee that expression will be successful (*see* **Note 4**). In the case of the T7 expression system this observation has been associated in many cases with a high level of expression of the target gene ***(15)***, which could still be optimised by changing the growth conditions (*see* **Subheading 3.4**).

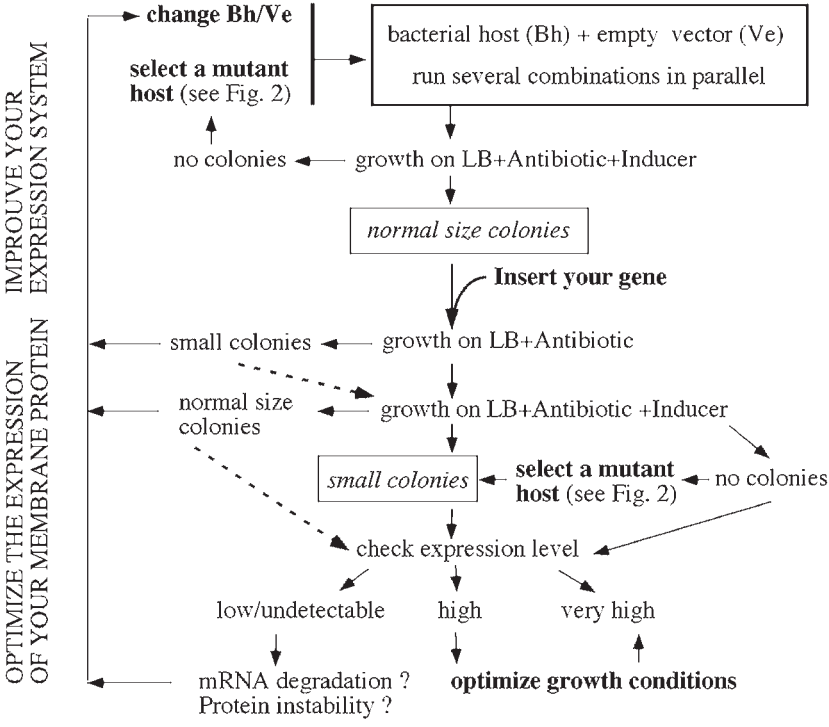

Fig. 1. General strategy for choosing the best combination of expression vector and bacterial host. The strategy relies on the fact that the expression system by itself should not be toxic and that the expression of the target gene is optimal when the growth of the cells after induction is only slightly reduced. This is reflected on agar plates containing the inducer by the formation of small size colonies.

In the third case, cells are unable to form colonies in presence of the inducer. This is very typical for membrane proteins but does not necessarily mean that the target gene is toxic. Our experience with the expression of the mitochondrial carriers and of the F1Fo ATP synthase is that some optimization is necessary to identify the right timing of induction or the appropriate strength of the promoter (*see* **Note 5**). Opting for host selection would be a sensible choice at this stage.

3.3.2. Obtaining Mutant Strains by Host Selection

A procedure for selecting a bacterial strain, which is better suited to expressing your target gene is illustrated in **Fig. 2**. In this example, the starter strain is BL21(DE3) and the expression plasmid, pGFP encodes the green fluorescent protein from *Aequora Victoria* ***(16)***. The steps are as follows:

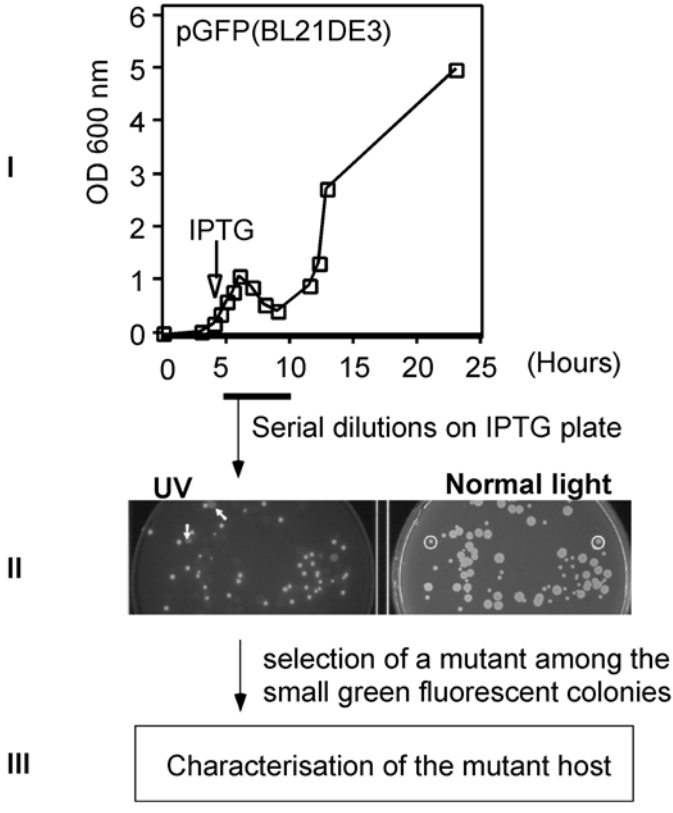

Fig. 2. Selection of mutant of *E. coli* BL21(DE3) using GFP as a gene reporter.

The green fluorescent protein is expressed in BL21(DE3). Three hours after induction of its expression, cells were diluted and plated out onto agar medium containing IPTG and ampicillin. The following morning, the plates were illuminated either with a UV lamp or with a normal lamp. Mutants that have kept the ability to express GFP form small fluorescent colonies while normal size bacterial colonies do not express GFP. White arrows and circles indicate the exceptions to rule.

1. Use a fresh colony to inoculate 50 mL of LB medium and induce expression (in this case, by adding IPTG to 0.7 m*M* final concentration). Monitor cell growth by measuring the optical density (OD) at 600 nm.
2. Typically, the OD decreases slightly after induction because some of the bacteria have immediately lysed. Cell growth does not recover until 1–3 h after the inducer is added. At the point just before the OD starts to increase again, cells should be diluted with LB medium or sterile water to make serial dilutions between 1:10 and 1:10,000. Cells are then immediately plated out on to LB-agar plates containing the appropriate inducer (e.g., 0.7 m*M* IPTG and antibiotic). Because the frequency of

viable cells and of mutants is variable, it is critical to make progressive dilutions of the culture in order to isolate individual colonies.

3. After overnight incubation at 37°C, two populations of cells should appear and in the case of GFP, the interesting mutants are immediately revealed under UV light. As illustrated in **Fig. 2**, most of the normal-sized colonies are not fluorescent. They represent mutants that have lost the ability to express GFP but keep ampicillin resistance. In some rare cases, some of these mutants like those indicated by a white arrow in **Fig. 2**, are poorly fluorescent. In contrast, almost all small colonies (except two of them highlighted by a white circle) are highly fluorescent, indicating high GFP expression. These are the mutant hosts to be isolated.
4. In order to have a strain you can work with, the cells must be cured of the plasmid. To do this, it is better to avoid the use of mutagenic compounds such as acridine orange; the simplest method consists of maintaining the selected strain in exponential phase for a week in LB medium without antibiotic or IPTG. Each day, you should make a serial dilution of the culture and plate out the cells on LB-agar plates containing IPTG but without antibiotic. After selection, the size of the colonies on IPTG plate can be considered as a signature of the mutant. On IPTG plates, mutant cells that have lost the plasmid will form normal size colonies and in the case of the GFP mutant, they will, of course, have lost their green fluorescence. To check that the mutation is in the bacterial host and not in the plasmid it is important to retransform the original plasmid into the isolated host and verify that the "colony size phenotype" on an IPTG plate is conserved.

3.4. Optimization of Growth Conditions

Optimization of expression conditions is essential for proteins that are difficult to express in high quantities and this can be done quite easily on a small scale. Temperature, type of medium, concentration of inducer, timing / length of induction, and degree of aeration can all influence cell growth and the yield of protein. The efficiency of aeration depends very much on the type of vessel used to grow the bacteria in, the speed of rotation, and the volume of culture. It is difficult and, therefore, not useful to test the influence of that factor in a small-scale screen. However, temperature, IPTG concentration, timing and length of induction can all be easily tested provided you have an Eppendorf thermomixer, or similar shaking / heating block.

A simple screen for growth conditions can be performed with 200-μL cultures, set up in 2-mL Eppendorf tubes. The number of tubes you prepare will depend on how many conditions you want to test at one time and how many different constructs you have. Eppendorf thermomixers contain 24 spaces, so 24 conditions at one temperature is the maximum you could try. If you have more than one shaker, it is convenient to test several temperatures in parallel.

A manageable setup is a total of 24 cultures split between two temperatures

Table 1
Optimization of Growth Conditions of the *E. coli* Culture

Temperature of induction: 37°C or 15°C		
Construct A (IPTG)	Construct B (IPTG)	
0.2 m*M*	0.2 m*M*	Induce at
0.7 m*M*	0.7 m*M*	OD_{600} = 0.6
1 m*M*	1 m*M*	
0.2 m*M*	0.2 m*M*	Induce at
0.7 m*M*	0.7 m*M*	OD_{600} = 1
1 m*M*	1 m*M*	

(room temperature [RT] and 37°C or RT and 15°C, for example). Two different constructs can be tested for a range of inducer concentrations and also timing and length of induction. A sensible range of conditions is illustrated in **Table 1**. The two constructs tested simultaneously are called A and B; 0.2 m*M*, 0.7 m*M*, and 1.0 m*M* refer to IPTG concentrations (*see* **Table 1**).

1. Prepare 5 mL master cultures of A and B by inoculating LB medium + antibiotic with a single colony from a fresh transformation (*see* **Note 6**). Incubate at 37°C and monitor cell growth by measuring the $OD_{600nm.}$
2. When the OD approaches 0.6, distribute 200-µL of each culture into 2-mL Eppendorf tubes; three for each construct in both the 37°C shaker and the 15°C shaker makes a total of 12 tubes. Induce expression by adding the appropriate volume of 100 m*M* IPTG.
3. Continue to measure the OD_{600} of the master cultures and repeat the previous steps once the optical density approaches 1.0 (*see* **Table 1**). Keep a sample of the uninduced master cultures. These will serve as controls for the SDS-PAGE to help you detect which band corresponds to the overexpressed protein.
4. Take 10-µL samples of the induced cultures after 3 h, 6 h and after overnight incubation.
5. Mix with 5X SDS-PAGE buffer and boil for 10 min to lyse the cells and denature the samples.
6. Spin them on a bench-top centrifuge for 10 min at 14,000*g* before analysing the supernatant on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) together with the control "uninduced" samples. You should end up with a total of 72 samples +2 controls. That is a rather colossal number if you want to run SDS-PAGE minigels so cut them down by being selective over the "induction time" samples. Only run the 3-h samples of the cultures grown at 37°C, run just the 6-h samples of those at grown at RT and take the overnight samples if the induction temperature was 15°C (*see* **Note 7**).

3.5. Large-Scale Expression and Harvesting of Cells

Once the optimum growth conditions have been identified in the small-scale screen, protein can be expressed in larger volumes. The level of expression will determine what volume of culture you should grow. It is wise to start with 1 or 2 L and perhaps grow two different constructs in parallel and then scale up later if necessary.

1. Make a 50-mL starter culture in a sterile 250-mL conical flask by inoculating medium + antibiotic with cells from a single colony. Grow overnight at 37°C with shaking.
2. The following morning prepare a chosen number of 2-L Erlenmeyer flasks with 500 mL of medium + antibiotic each. Prewarming the medium to 37°C will speed up the cell growth, but is not essential.
3. Inoculate each 500 mL with 5 mL of starter culture.
4. Grow the cells at 37°C with shaking at about 150–200 rotations per min. Monitor cell growth by measuring the OD_{600} every 30 min.
5. When the induction time is up, harvest the cells by centrifuging at 2000*g* for 15 min at 4°C (*see* **Note 8**).

3.6. Expression Conditions for UCP1

It has not been possible to find conditions where the full-length UCP1 protein can be both highly expressed and correctly inserted into the plasma membrane; in C41(DE3) the recombinant protein accumulates in high amounts as inclusion bodies instead. Our strategy for obtaining pure, functional protein has been to denature and refold these inclusion bodies. However, there are a number of difficulties associated with refolding of membrane proteins. The absence of a native membrane to insert into is a potential problem and the chances of misfolding are certainly likely to increase according to the size of the protein and number of transmembrane domains. Our logic was to isolate each of the three repeats of UCP1 as individual constructs. With each one possessing two transmembrane spans and a 40-residue loop, the chances of correct refolding are higher than for the full-length protein.

The full-length UCP1 and the fragments of the protein were cloned into a pET like vector and a T7 based high copy number plasmid (gift of M. Runswick) derived from pMW7 ***(13)***, which both give protein with a 6X histidine tag. Depending on the vector, the tag is either at the N-terminus and separated from the protein by a Tev (Tobabcco Etch Virus) protease cleavage site, or is C-terminal and not cleavable. As a selectable marker, both vectors confer ampicillin resistance and a T7-*lac* promoter initiates transcription in the presence of IPTG.

1. C41(DE3) cells that harbor the expression plasmid are grown at 37°C in LB medium until the OD_{600} reaches 0.6.

2. Overexpression is induced by adding IPTG to a final concentration of 0.7 m*M*. Protein yields are equally good whether cells are induced at 37°C for 3 h or at 25°C overnight.
3. Cells are harvested by centrifugation and lysed at 4°C by passing them twice through a French pressure cell. Unbroken cells are isolated by spinning the lysate at 600*g* for 15 min.
4. The supernatant is recentrifugated at 10,000*g* for 20 min to isolate the inclusion bodies.
5. A homogeniser is used to resuspend the inclusion bodies in Inclusion body buffer to a concentration of 30 mg total protein per mL. Comprising mostly overexpressed protein (*see* **Fig. 3**), the yield of inclusion bodies is typically 250–300 mg L of culture.

Notes

1. The following Web resources are useful in planning and implementing the expression of membrane proteins:
   SMART (protein domain database)
   http://smart.embl-heidelberg.de/
   Jpred (secondary structure prediction)
   http://jura.ebi.ac.uk:8888/
   EMBL Protein Purification and Expression Unit
   http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/frames_which_vector_ext.htm/
   ExPasy
   http://www.expasy.ch/
2. With such a large variety of vectors to test out, it is not difficult to get overloaded so keep the number of constructs well within a limit you can realistically manage and keep the cloning steps as simple as possible (try to use the same restriction sites for all the constructs, for example). For more details on the types of vectors available, the web page of the EMBL Protein Purification and Expression Unit is a good resource. *http://www.emblheidelberg.de/ExternalInfo/geerlof/draft_frames/frame_which_vector_ext.htm/*
3. We have frequently observed that some expression systems are so leaky (i.e., overexpression occurs before inducer is added) that even in the absence of inducer on the plates, cells are unable to form regular sized colonies. In severe cases, such as the mitochondrial ADP/ATP carrier or the B-subunit of the *E. coli* ATP synthase expressed in BL21(DE3) with the pMW7 vector, cells form pinhead sized colonies which do not grow in liquid medium ***(15)***. In such cases, a new combination of vector and host needs to be found.
4. If in spite of attempts to optimize growth conditions, the expression level is still very low you should consider investigating the stability of both the mRNA and the target protein. Predictive programmes allow the determination of secondary structure susceptible to RNA degradation ***(11)*** and of the half-life of your protein in various expression systems (*see* ExPasy web site). Silent point mutations may help in

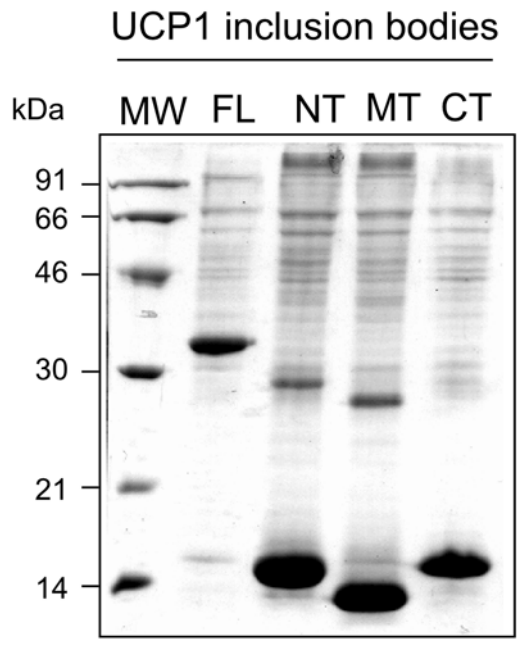

Fig. 3. Analysis by SDS-PAGE of the inclusion bodies preparation of four different constructs of rat UCP1: the full length UCP1 (FL), its N-terminal third (NT), middle third (MT), and C-terminal third (CT). Ten micrograms of inclusion bodies were loaded on the gel. The gel was stained using Coomassie blue dye.

stabilizing mRNA and using a protein fusion tag (e.g., Glutathione S transferase, GST) has also been known to increase protein stability.

5. Obtaining stable, correctly folded protein does not stop at translation, membrane insertion is necessary and the cell may need time for metabolic adaptation such as lipid synthesis and membrane proliferation. Expression of the b-subunit of the *E. coli* ATP synthase is a very interesting example. The BL21(DE3) strain was intolerant to the pMW7(Ecb) plasmid even before induction of expression. Equally, expression in C41(DE3) was toxic to the cell and the b subunit was somehow misfolded. A specific mutant host, C43(DE3) was then selected as described in **Subheading 3.3.2.** and expression gave no signs of toxicity. Upon induction of expression, the bacterial host produced an internal membrane network in which the b-subunit was concentrated ***(17)***. This is why we strongly recommend selecting mutant hosts in order to specifically optimise the expression system for your membrane protein.
6. When inoculating a starter culture for expression, it is wise to start from a fresh colony rather than a liquid culture. There is a possibility that the cells may lose the plasmid or some recombination may occur. Ideally, you should start from a plate of freshly transformed cells.

7. It is important to bear in mind that good expression does not necessarily mean the final yield of purified protein will be high enough. It is worth extending the screen and testing for purification and cleavage (in case the protein has a tag or fusion protein) before deciding which vector is best. It is not uncommon that a tagged protein can be purified in high amounts but precipitates as soon as it is cleaved from the fusion partner!
8. When growing large cultures, if you want to induce the overexpression at a temperature lower than 37°C, it is important to lower the temperature of the incubator well in advance (1 h–30 mins) of the point at which you want to add the inducer. The incubator may cool down quickly, but the cultures will take some time to reduce in temperature. If you overshoot the point at which you should start cooling the incubator, put the flasks at 4°C until they reach the correct temperature.

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4

Expression of Membrane-Bound Iron–Sulfur Proteins

Dirk Schneider, Christian L. Schmidt, and Andreas Seidler

1. Introduction

Overexpression of membrane-bound proteins to obtain milligram quantities for biochemical and biophysical studies is usually a difficult task, especially for those proteins that span the membrane several times ***(3)***. An additional difficulty arises when these proteins need to incorporate organic or inorganic cofactors. In order to obtain a functional protein, this requires a host that is able to produce this cofactor or the cofactor has to be added to the growth medium and subsequently taken up by the host cells. Alternatively, the protein can be expressed as inclusion bodies and then refolded in the presence of the cofactor(s), a strategy successfully applied for bacteriorhodopsin and the light-harvesting complex of higher plants ***(3)***.

There are few examples of successful overexpression of membrane-bound FeS proteins; most of them are *Escherichia coli* (*E. coli*) proteins overproduced in *E. coli*. In fact, these proteins were globular subunits of large membrane-associated complexes like NADH dehydrogenase or fumarate ***(2)*** reductase ***(19)***.

The Rieske FeS protein is a membrane-associated compound of the electron transport chain of photosynthesis and respiration ***(4)***. It is a subunit of the cytochrome *bc* complex in mitochondria, chloroplasts, and bacteria. The protein consists of an N-terminal transmembrane helix and a globular domain located on the P-side (intermembrane space/thylakoid lumen/periplasm) of the membrane carrying a 2Fe2S cluster. In addition, this protein contains a disulfide bridge, which is known to be important for the stability of the FeS cluster. Because of the important role of this protein in electron transfer ***(5)***, it has been studied intensively in recent years. Successful overexpression of the Rieske protein with incorporated FeS cluster was reported only for those proteins

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derived from thermophilic *archea* ***(5,12)*** but not for those from mesophilic organisms. However, a truncated version of this protein from a mesophilic cyanobacterium was heterologously expressed and a significant fraction was found to carry a 2Fe2S cluster ***(6)***. In this chapter, we describe methods used for overexpression of three full-length Rieske FeS proteins from the cyanobacterium *Synechocystis* PCC 6803. Two of them, PetC1 (product of ORF sll1316) and PetC2 (product of ORF slr1185), are rather similar to the Rieske protein found in cytochrome b_6f complexes, whereas the other (PetC3, product of sll1182) is quite different in both sequence and size. The PetC1 protein had to be refolded and the iron–sulfur cluster had to be reconstituted in vitro after purification of the protein ***(13)***, the two other proteins were also obtained in a membrane-bound form carrying the 2Fe2S cluster (*see also* **ref.** ***14)***.

2. Materials

1. pRSET6a expression vector, pLysE plasmid (Novagene).
2. *E. coli* strains DH5α, BL21(DE3).
3. Oligonucleotide primers for polymerase chain reaction (PCR).
4. Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase.
5. Plasmid miniprep kit, DNA purification kit (for purification of DNA from agarose gels).
6. LB medium containing 100 μg/mL ampicillin with or without 50 μg/mL chloramphenicol.
7. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
8. PBS buffer: 137 m*M* NaCl, 2.7 m*M* KCl, 12.8 m*M* Na_2HPO_4, 1.76 m*M* KH_2PO_4, pH 7.2.
9. Sonicator.
10. Proteinase inhibitors:phenylmethylsulfonyl fluoride (PMSF), p-tosyl-L-lysine chloromethyl ketone (TLCK).
11. Magnesium chloride.
12. DNase I (Boehringer).
13. 50 m*M* Tris-HCl, pH 8.3.
14. Wash buffer: Phosphate-buffered saline (PBS) containing 25% sucrose, 1% Triton-X-100, 5 m*M* ethylene diamine tetraacetic acid (EDTA).
15. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.

3. Methods

The following paragraphs describe

1. The construction of expression plasmids for the overexpression of three different *Synechocystis* Rieske proteins;
2. Conditions for successful protein expression; and
3. Characterization of the expressed proteins.

3.1. Expression Vector

In general, the conditions for successful overexpression of membrane proteins cannot be predicted and must be determined empirically. Many membrane proteins seem to behave differently and the expression levels depend on many factors as discussed in ***(3)***. For the expression of proteins in *E. coli*, many different plasmids and promoter systems have become available in the recent years. The different features of these systems strongly influence the expression of foreign proteins in *E. coli*. Therefore, each system has to be optimized either to obtain a functional membrane protein incorporated into *E. coli* membranes or to produce large quantities of insoluble protein, localized in inclusion bodies.

In recent years, it turned out that the integration of proteins into the *E. coli* membrane is an important and rate-limiting factor ***(1–3)***. In the case of polytopic membrane proteins, the use of a low-copy-number plasmid in combination with a weak promoter may be most promising, because low-level bacterial expression could result in a higher degree of integration of the protein into the cytoplasmic membrane of *E. coli* ***(3)***. However, in our hands, the use of the strong ψ10 promoter of the bacteriophage T7 for the Rieske FeS protein was most successful.

3.1.1. pRSET6a

The pRSET family of expression plasmids are T7 promoter-based expression vectors derived from pBluescript ***(15)***. They are high-copy-number vectors featuring translational start and stop elements and a multiple cloning site. Because of the T7 promoter, which is not recognized by the *E. coli* RNA polymerase, the system is "off" in *E. coli* strains not carrying the T7 RNA polymerase gene. In strains bearing this gene (like BL21 [DE3]) the T7 RNA polymerase gene is under the control of the *lacUV5* promoter and after promoter induction, proteins are usually produced at a very high level. In the uninduced state, the gene of interest is transcribed at a low level, even without additional induction of the promoter. This allows a moderate level of protein expression without significant inhibition of cell growth. The coexpression of T7 lysozyme from the plasmids pLysE or pLysS allows more stringent control of protein expression, because lysozyme is a natural inhibitor of the T7 RNA polymerase ***(17)***.

The described characteristics make the pRSET vectors a suitable system for the large scale production of proteins (after induction with IPTG) and for low-level expression (without induction), which can result in the functional expression and folding of a membrane protein and its incorporation into the *E. coli* cell membrane.

3.1.2. Preparation of Genomic DNA From Synechocystis PCC6803

Usually, the purification of genomic DNA from the cyanobacterium *Syechocystis* involves several steps. Briefly, after cell disruption the DNA is extracted by phenol/chloroform and afterward further purified. For the purpose of performing PCR, the DNA preparation does not have to be very pure, and quicker methods are effective. In ***(8)***, the authors describe a relatively fast method for the small-scale isolation of genomic DNA for PCR applications. Trying to use the simplest approach, we harvested 1.5 mL of cyanobacterial cells (*see* **Note 1**) by centrifugation, resuspended the cells in 100 μL TE buffer ***(11)***, and incubated the cells for 2–3 min at 100°C. Afterwards, the cells were sedimented again and the supernatant was transferred to a new reaction tube. 5–10 μL of this solution were used as DNA template in PCRs.

3.1.3. DNA Cloning

Molecular cloning was carried out using standard techniques described in ***(8)***. The three full-length *Synechocystis petC* coding regions were amplified individually by PCR. For cloning purposes, an *NdeI* site was introduced at the 5' end of the ORF including the translation start codon ATG, and a *BglII* or a *BamHI* site was introduced at the 3' end of the ORFs. The PCR-amplified DNA fragments were cleaved with *Nde*I and *BglII* or *BamHI*, respectively, and cloned into the appropriate restricted expression plasmid. After transformation of the plasmids into *E. coli* DH5α, the cells were grown overnight on standard LB plates containing 100 μg ampicillin/mL. Single colonies were tested afterwards for the insertion of the *petC* coding regions into pRSET6a. In order to exclude any error introduced by the PCR, the insert of one clone of each expression plasmid was finally sequenced.

3.2. Protein Expression

The level of expression, the localization of a membrane protein in *E. coli* (soluble, inclusion bodies, membrane), and its structure (folded vs unfolded) depends on many factors. It should be noted that the optimal expression conditions have to be determined for each individual protein by testing a number of important parameters like the *E. coli* strain, the growth medium, the expression temperature, the expression time, the addition of supplements, and so on.

3.2.1. E. coli Strain, Transformation, and Induction

Because the gene of interest in the pRSET plasmid is under the control of the T7 promoter, a strain carrying the λDE3 lysogen has to be chosen for protein expression. One of the most commonly used strains is *E. coli* BL21 (DE3), which was also used in this study (*see* **Note 2**). The cells were transformed with

the individual expression plasmids by standard techniques ***(11)***, plated on LB plates that contain the appropriate antibiotics, and incubated overnight at 37°C.

3.2.2. Expression of PetC1

1. Inoculate 10 mL LB medium containing 100 μg/mL ampicillin with a fresh single colony and shake the culture for 6 h at 37°C.
2. Centrifuge this culture to sediment the cells at 3000*g* for 10 min.
3. Resuspend the cells in 500 mL fresh medium.
4. Incubate the culture for 15 at 37°C with continuous shaking without addition of IPTG.
5. Harvest the cells for further protein purification and characterization.

3.2.3. Expression of PetC2 and PetC3 (see ***Note 2***)

1. Select a fresh single colony and inoculate 10 mL LB medium containing the appropriate antibiotics (100 μg/mL ampicillin plus or minus 50 μg/mL chloramphenicol).
2. Let the cells grow for 6–8 h at 37°C.
3. Sediment cells by centrifugation for 10 min at 3000*g* and resuspend the sediment in 5mL fresh medium containing the appropriate antibiotics.
4. Store cells overnight at 4°C.
5. The next morning, inoculate 500 mL fresh medium with 5 mL of cells (*see* **step 4**) and shake at 37°C.
6. At an OD_{600} = 0.6, induce protein expression by addition of 0.5 m*M* IPTG from a freshly prepared stock solution.
7. Harvest cells after 3–4 h for protein purification.

3.2.4. Cell Harvest

1. After protein expression, harvest cells by centrifugation (5000*g*, 10 min) and resuspend the cells in 20 mL PBS buffer.
2. The cells can be frozen at −20°C at this step without any degradation of the overexpressed proteins. Because of the freezing step, some cells are already disrupted and DNA is released. This can result in a slimy, tacky pellet if the cells were not resuspended before. Therefore, we recommend resuspending the cells before freezing.

3.3. Protein Extraction

3.3.1. Cell Disruption

1. Add 1 m*M* PMSF and 0.1 m*M* TLCK to the resuspended expression cultures.
2. Break the cells by sonication (*see* **Note 3**).
3. Add 25 m*M* $MgCl_2$ and 0.1 mg/mL DNase. Incubate the broken cells for 30 min at 37°C (*see* **Note 4**).

3.3.2. Fractionation of E. coli Cells

Possible strategies for the purification of the individual Rieske proteins from *E. coli* after overexpression are shown in **Fig. 1**. The strategy of choice depends

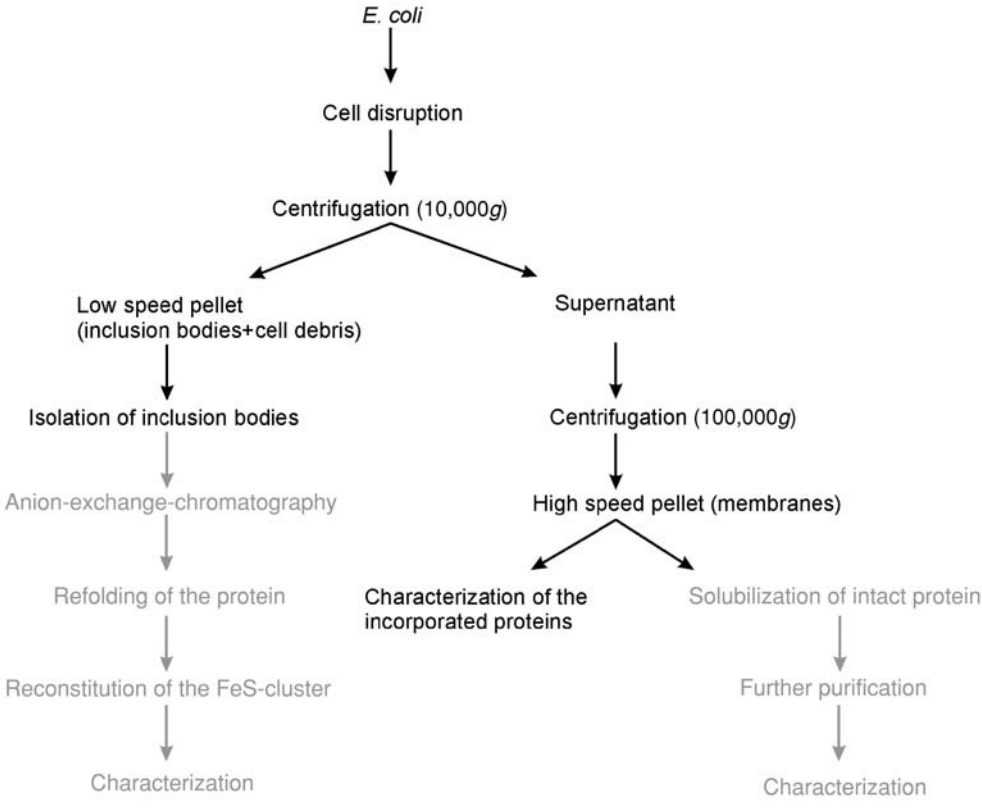

Fig. 1. Flowchart of the procedure used to purify the Rieske proteins overexpressed in *E. coli*. Black steps are described in detail in the text. Gray steps indicate possible further steps not described in this chapter.

on the localization of the expressed protein and the structure of the protein (native vs inclusion body).

3.3.2.1. Inclusion Body Isolation

1. Centrifuge the broken cells at 10,000*g* for 10 min.
2. Save the supernatant for the membrane preparation (*see* **Subheading 3.3.2.2.**).
3. Resuspend the sediment (inclusion bodies and cell debris) in 10 mL wash buffer with 1m*M* PMSF and 0.1 m*M* TLCK.
4. Centrifuge for 10 min at 10,000*g* and discard supernatant.
5. Repeat **steps 3** and **4** twice.
6. Resuspend the sediment in 20 mL water and centrifuge as in **step 4**. Repeat this step once.

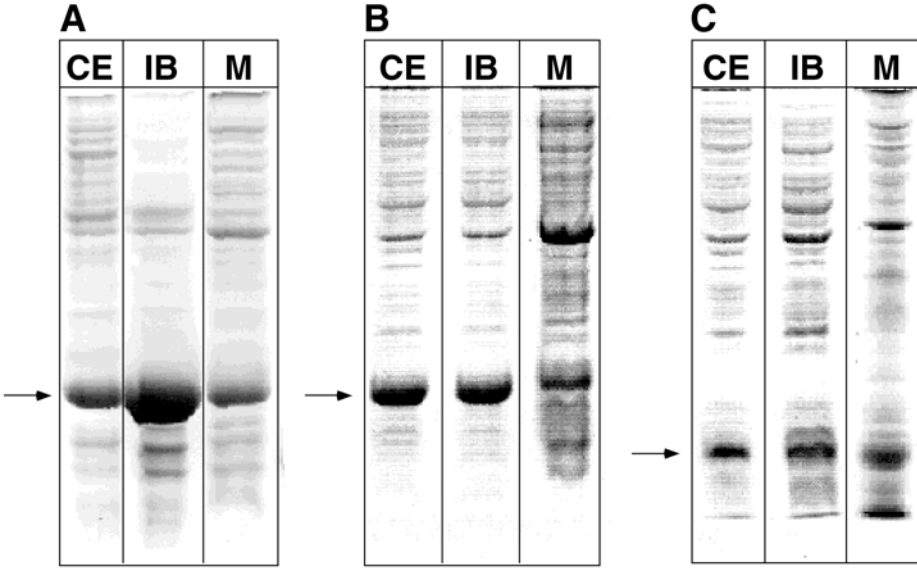

Fig. 2. Localization of the overexpressed Rieske proteins PetC1 **(A)**, PetC2 **(B)**, and PetC3 **(C)** in *E. coli.* CE, cell extract; IB, inclusion bodies; M, membranes.

7. Resuspend the pellet in 1 mL PBS buffer with 1 m*M* PMSF and 0.1 m*M* TLCK (*see* **Note 5**).
8. The purified inclusion bodies can be stored for further purification at −20°C (*see* **Note 6**).

3.3.2.2. Membranes

1. Centrifuge the supernatant from **step 2** (*see* **Subheading 3.3.2.1.**) in an ultracentrifuge for 30 min at 100,000*g*.
2. Resuspend the sediment in 500 μL 50 m*M* Tris-HCl, pH 8.3, 1 m*M* PMSF, 0.1 m*M* TLCK.
3. This membrane fraction can be stored at −20°C.

3.4. Characterization

The success of protein overexpression and purification can be easily determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The described method leads to a highly enriched Rieske protein (*see* **Fig. 2**); in the case of the *Synechocystis* PetC3 protein no additional purification step is necessary after isolation of the inclusion bodies (*see* **step 3.3.2.2.** and **Note 5**).

3.4.1. Rieske-Type EPR Signals

The Rieske protein was originally characterized by Rieske et al. ***(19)*** by the existence of the typical EPR signal of the iron–sulfur cluster. To check if the individual *E. coli* fractions (membranes, inclusion bodies) show a new EPR signal typical for the Rieske iron–sulfur cluster EPR spectra were recorded on a Bruker EPR200 spectrometer. The sample temperature was kept at 15 K by a helium cryostat. Typical parameters for a measurement were: 6.3 mW microwave power, 10 G modulation amplitude, and 9.44 GHz microwave frequency. The samples were completely reduced by the addition of a few grains of $Na_2S_2O_4$, partly reduced by addition of 5 m*M* sodium ascorbate, and completely oxidized by addition of 5 m*M* CeO_2 (*see* **Note 7**). For the first measurements, the protein samples were measured undiluted.

By this method it could be shown that in *E. coli* membranes of the strains overexpressing the *Synechocystis* PetC2 and PetC3 proteins, the typical Rieske EPR signal can be observed indicating the presence of a correctly folded protein in the membrane with an incorporated cluster (*see* **Fig. 3**). Whereas the iron–sulfer clusters of both PetC2 and PetC3 were completely reduced after addition of $Na_2S_2O_4$, the PetC3 EPR signal was almost not observable after partial reduction with ascorbate in contrast to PetC2. Under oxidizing conditions, a Rieske EPR signal was observed in none of the samples (*see* **Note 8**).

In the case of the PetC1 protein, a fraction of the protein is incorporated in *E. coli* membranes, but no typical EPR signal was detected. In order to obtain a folded protein with incorporated iron–sulfur cluster, this protein has to be folded and reconstituted in vitro ***(6,13)***.

4. Notes

1. The cultivation medium and conditions are described in ***(10)***.
2. As mentioned in **Subheading 3.1.**, the pRSET vector has some background expression even without extra induction of the expression by IPTG. Although this can be used for low-level expression of proteins (like described here for PetC1), this can also lead to cell death of the transformants if the protein is very toxic for *E. coli*. In the case of the *petC3* gene, no protein expression was achieved using BL21(DE3). Even the cotransformation of the helper plasmid pLysS, from which lysozyme is constitutively expressed at a certain level, is not sufficient to block the protein expression strongly enough. PetC3 can only be overexpressed successfully in a BL21(DE3) strain harboring the plasmid pLysE.
3. Alternative methods can be utilized for cell lysis including the use of a French press, treatment with lysozyme, or several cycles of freezing and thawing. BL21(DE3) cells harboring the pLysS or pLysE plasmids can easily be lysed by freezing once and incubation of the cells afterward at 37°C. The freezing step

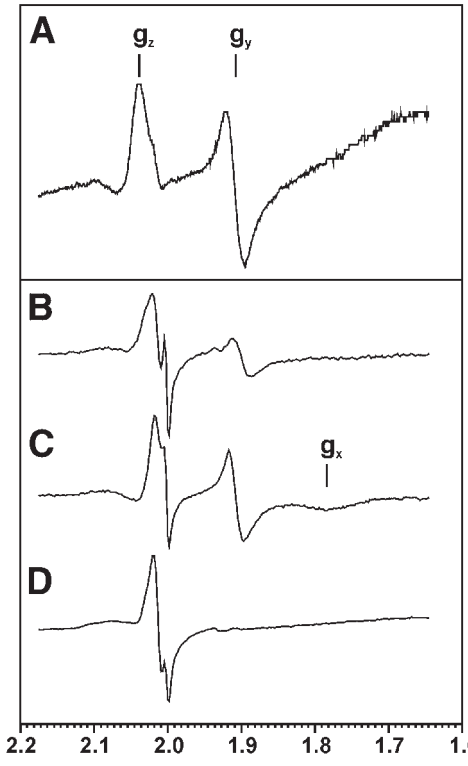

Fig. 3. EPR spectra form *E. coli* cell membranes containing PetC2 **(B)** and PetC3 **(C). (A)** Shows the EPR signal from the isolated and in vitro reconstituted PetC1 protein ***(13)***, and in **(D)** a control of *E. coli* cell membranes is shown. The positions of the $g_{y,z}$- values are indicated in **(A)**. The g_x-value could only be determined accurately for the PetC3 protein **(C)**.

In all membrane protein preparations an additional EPR signal with a g-value of 2.02 was detected, most likely caused by the presence of small amounts of copper. Beside this signal additional signals, typical for the presence of Rieske iron–sulfur clusters were detected. The samples were completely reduced by addition of $Na_2S_2O_4$. The protein concentration was 40 mg/mL.

breaks some cells, which leads to the release of internal lysozyme produced by the *lys* gene. The addition of 25m*M* $MgCl_2$ is recommended.

4. The released nucleic acids often result in the formation of a slimy, sticky pellet. The further fractionation of this pellet (cell debris, inclusion bodies, and membranes) can be more problematic under such conditions. The treatment of the sediment with DNase (and RNase) can improve further purification steps.

5. Although the overexpressed protein is highly enriched in inclusion bodies, usually the protein is not completely pure and further purification steps are required. Because PetC3 is virtually insoluble at this pH, the pellet can further treated with 50 m*M* phosphate buffer (pH 12.0). By this step, almost all of the *E. coli* proteins are solubilized, whereas PetC3 can still be sedimented by centrifugation. This additional washing step results in an electrophoretically pure PetC3 protein without further purification.
6. For further purification of the protein, we solubilize the final inclusion body pellet in 50 m*M* Tris-HCl pH 8.3 + 8 *M* urea and filtered this protein solution through a 0.45 μ*M* filter. After the isolation of the inclusion bodies, the protein is already highly enriched (*see* **Fig. 2**). In the case of the PetC3 protein, the inclusion body fraction is already electrophoretically pure. The proteins PetC1 and PetC2 can be further purified by one anion-exchange chromatography step and refolded and reconstituted in vitro. Because these steps are beyond the scope of this article, the interested reader is referred to ***(6,14)***.
7. Usually, $K_3Fe_2(CN)_6$ is used for the oxidation of Rieske proteins. Because the iron shows a very high electron paramagnetic resonance (EPR) signal, the usage of CeO_2 is recommended.
8. The midpoint potential of the Rieske protein strongly depends on the amino acids surrounding the iron–sulfur cluster as discussed in ***(5,7)***. By this theoretical analysis of a given amino acid sequenced, the range of the midpoint potential of the Rieske protein of interest can already roughly be estimated and its EPR characteristics after complete or partial reduction can be predicted.

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2

Dihydroorotate Dehydrogenase of *Escherichia coli*

Kaj Frank Jensen and Sine Larsen

1. Introduction

1.1. Different Types of Dihydroorotate Dehydrogenases (DHODs)

Dihydroorotate dehydrogenase (DHOD) catalyzes the fourth reaction in the pathway for *de novo* synthesis of UMP and forms the 5,6-double bond of the pyrimidine base. In this reaction, two electrons and two protons are transferred from dihydroorotate to an electron acceptor that varies between different types of the enzyme. Sequence alignments have shown that all DHODs contain a polypeptide chain that is encoded by a *pyrD* gene. This polypeptide forms the catalytic core structure, folding into an $(\alpha/\beta)_8$-barrel. The active site, which contains a tightly bound molecule of flavin mononucleotide (FMN), is formed by loops that protrude from the top of the barrel (e.g., **ref. *1***). The first half reaction, in which the enzyme is reduced and dihydroorotate is oxidized to orotate, is initiated by binding of dihydroorotate at the *si*-side of the isoalloxazine ring of FMN ***(2)*** and, after abstraction of a proton from the 5'-position of dihydroorotate by a cysteine or a serine residue in the enzyme, a hydride ion is transferred to FMN from the 6-position of the substrate ***(3,4)***. The first half reaction is common to all DHODs, but different types of DHODs deviate from each other in quaternary structure, subcellular location, and use of electron acceptors to reoxidize the reduced enzyme in a second half reaction ***(5)***.

1.1.1. The Soluble Class 1 DHODs

The class 1 DHODs are soluble proteins. Two types have been identified. Class 1A DHODs are dimeric proteins able to use fumarate as electron acceptors. The enzymes are found in milk fermenting bacteria like *Lactococcus lac-*

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tis ***(6,7)*** and *Enterococcus faecalis* ***(8)***, in the anaerobic yeast *Saccharomyces cerevisiae* ***(9,10)*** and in some eukaryotic parasites ***(11,12)***. The enzyme from *L. lactis* (DHODA) has been studied in considerable detail and the crystal structure has been solved of the free enzyme and as a complex with the product orotate ***(1,2)***.

Class 1B DHODs are heterotetrameric enzymes that use NAD^+ as electron acceptor ***(13)***. The occurrence is restricted to Gram positive bacteria. The closely related strains *L. lactis* ***(14)*** and *E. faecalis* ***(15)*** have both a class 1A and a class 1B DHOD ***(6)***, but species of *Bacillus* ***(16,17)*** and *Clostridium* ***(4,18)*** only possess a class 1B enzyme. The protein from *L. lactis* (DHODB) has been studied in detail ***(13)*** and the crystal structure has been solved for the free enzyme and as a complex with the product orotate ***(19)***. Two of the subunits are encoded by the *pyrDb* gene, and together they form a dimeric protein like DHODA. Associated with this catalytic core are two tightly bound electron transfer subunits, which are encoded by the *pyrK* gene and protrude from the catalytic dimer like two moose horns. The PyrK polypeptides belong to the ferredoxin reductase superfamily. They have flavin adenine dinucleotide (FAD) and a [2Fe-2S] cluster as cofactors and are engaged in the channeling of electrons to NAD^+ ***(13,19)***.

Other types of soluble DHODs exist. For instance, a class 1B-like DHOD able to use molecular oxygen, but not NAD^+, has been found in *Lactobacillus* and is devoid of an electron transfer subunit ***(20,21)***. In addition, the archaeon *Sulfolobus solfataricus* has a class 1B-type DHOD associated with an iron-sulfur cluster protein different from PyrK. The electron acceptor preferences of this protein is unknown ***(22)***.

1.1.2. The Membrane Associated Class 2 DHODs

The membrane associated class 2 DHODs use quinones of the respiratory chain as electron acceptors. They are found in Gram negative bacteria like *E. coli* ***(23)*** and *Helicobacter pylori* ***(24)***, where they are associated with the cytoplasmic membrane, and in most eukaryotic organisms, where are anchored in the inner mitochondria membrane ***(25)***. The class 2 enzymes are monomeric proteins with a strong tendency to aggregate ***(26,27)***. The core part of the enzymes, with the active site, forms an $(\alpha/\beta)_8$-barrel structure similar to the structure of the class 1 enzymes ***(28,29)*** although the sequence similarity between the two classes of DHODs is very low, 12–20% identity ***(5,30)***. The polypeptide chains of all class 2 enzymes are extended in the N-terminal relative to the class 1 enzymes (*see* **Fig. 1**). In bacteria this extension sequence is just a little more than 40 amino acid residues. In the *E. coli* enzyme (DHODC) it forms a separate helical domain with a hydrophobic cavity between two of the helices, located at the side of the core domain ***(28)***. The small N-terminal

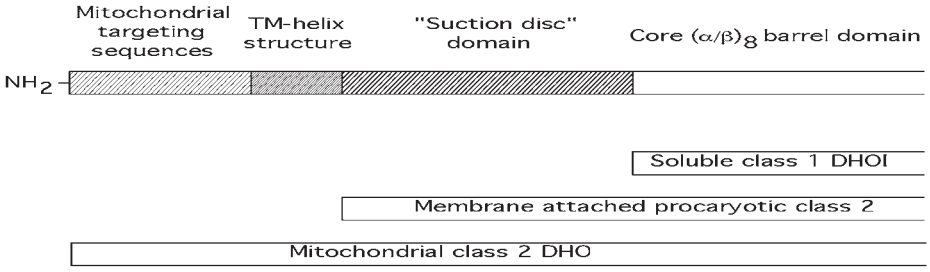

Fig. 1. Schematic representation of the functional roles of sequence elements in the polypeptide chains of different dihydroorotate dehydrogenases. *See* main text for further explanation.

domain enables DHODC to use respiratory quinones as electron acceptors (menaquinone appears to be the physiological electron acceptor of DHODC ***[31]***) and is essential for the association of the enzyme to the membrane by a mechanism that essentially is unknown ***(28)***. We call the domain "a suction disk", but do not know if the quinones, which bind to this domain, are involved in membrane association through their long hydrophobic tails.

The mitochondrial class 2 DHODs share the "suction disk domain" with the enzymes of prokaryotic origin ***(29)***, but the N-terminal extensions of the mitochondrial enzymes are longer than their prokaryotic counterparts, as they contain a short segment of 16–20 amino acid residues which (from the sequence) is predicted to form a transmembrane helix just upstream of the "suction disk structure" and carry sequences that target the proteins for import in mitochondria ***(25)*** (*see* **Fig. 1**).

Our current procedure for overexpression, purification, and crystallization dihydroorotate dehydrogenase from *E. coli* consists of the following major steps:

a. Growth of cells and over-production of DHOD from a plasmid encoded, inducible gene.
b. Disruption of cells by ultrasonic treatment and release of the enzyme from membranes by Triton X-100 in the crude extracts.
c. Chromatography on a DE-52 anion exchange column in the presence of Triton X-100.
d. Hydrophobic interaction chromatography on a column of Phenyl Sepharose and elution with Triton X-100.
e. Chromatography on a second anion exchange column to remove the detergent.
f. Crystallization with sodium formate as precipitant.

The procedure yields about 20 mg DHODC per liter bacterial culture ***(27)***. The crystal structure was published by Nørager et al. ***(28)***.

2. Materials

1. *The expression plasmid:* The expression vector pAG1 ***(27)*** is a derivative of the ampicillin resistance plasmid pUHE23-2 ***(32)***. It carries the 336 codons reading frame of the *E. coli pyrD* gene, encoding DHODC, cloned behind the strong $T7_{A1/04/03}$ promoter, which is a synthetic derivative of the $T7_{A1}$ early promoter and contains two operator sites for binding the LacI repressor.
2. *Bacterial strains:* The *E. coli* strain SØ6645 (*araD139*Δ*(ara-leu)7679 galU galK*Δ*(lac)174*Δ*pyrD(BssHII-MluI::Km*^{r}) [F' *proAB lacI*^{q}*Z*Δ*M15Tn10*] overproduces the LacI repressor from the *lacI*^{q} gene on the episome and is deleted for the promoter proximal part of the chromosomal *pyrD* gene ***(7)***. Strain SØ6735 (*rph-1 metA recA56 srl*::Tn*10*) [F' *proAB lacI*^{q1}*Z*::Tn*5*] ***(28)*** is derivative of the methionine requiring strain DL41 previously used for production of selenomethionine substituted proteins ***(33)***.
3. Preswollen diethyl aminoethyl cellulose (DE-52) is available from Whatman Ltd. (Maidstone, England).
4. Phenyl Sepharose® CL-4B is available from Pharmacia LKB (Uppsala, Sweden).
5. LB-broth: 10 g Bacto® Tryptone (Difco, Detroit, MI), 5 g yeast extract (Oxoid Ltd., Basington, UK) and 5 g NaCl per liter of ion exchanged water. If needed, the pH was adjusted to 7.0 by addition of NaOH before autoclaving ***(34)***.
6. Solution A: 20 g $(NH_4)_2SO_4$, 75 g $Na_2HPO_4.2H_2O$, 30 g KH_2PO_4, and 30 g NaCl per liter.
7. Solution B: 20 mL of 1 *M* $MgCl_2.6H_2O$, 2 mL of 0.5 *M* $CaCl_2.2H_2O$, and 3 mL of 10 m*M* $FeCl_3.6H_2O$ per 9 L ***(35)***.
8. (A +B) basal salt medium: Mix together autoclaved Solution A and Solution B, one part A to nine parts B.
9. Supplements are added from sterile solutions that had been autoclaved separately at 100°C, glucose as a 20% solution, amino acids at a concentration of 5 mg/mL and uracil 2 mg/mL. Ampicillin (Sigma, St. Louis, MI) and isopropyl-β-D-thiogalactoside (IPTG; Bingswood Industrial Estate, Whaley Bridge, UK) are added as solid material.
10. Buffer A: 5 m*M* sodium phosphate pH 7.0 containing 0.25 m*M* ethylenediamine tetraacetic acid (EDTA) (*see* **Note 1**).
11. Buffer B: 5 m*M* sodium phosphate pH 7.0, 0.25 m*M* EDTA, 5 m*M* $MgCl_2$, 0.1% Triton X-100 (Sigma).
12. Buffer C: 50 m*M* sodium phosphate, pH 6.2, containing 0.1 m*M* EDTA, and 0.1% Triton X-100.
13. Buffer D: 50 m*M* sodium phosphate pH 7.0 containing 0.1 m*M* EDTA.
14. Centrifugation spin columns (Amicon, Centriprep®).

3. Methods

3.1. Growth and Harvest of Cells

Strain SØ6645 transformed with the expression plasmid pAG1 was grown at 37°C in LB-broth medium containing ampicillin (100 mg/L). To ensure a high production of DHODC we always use freshly transformed cells (*see* **Note 2**). A preculture (100 mL) is inoculated in the morning with 4–5 single colonies from

a fresh transformation agar plate and grown to an OD_{436} of circa 0.5, when it is cooled in an ice bath. The preculture is stored at 4°C overnight and diluted into 2 L of prewarmed medium on the morning of the next day (*see* **Note 3**).

The culture is grown with vigorous aeration by shaking. IPTG (0.5 m*M*) is added at OD_{436} = 0.7–1.0 to induce expression of the *pyrD* gene and growth is continued overnight, while the culture reaches stationary phase at an OD436 about 5 (*see* **Note 4**). Cells are harvested by centrifugation, washed with 0.9% sodium chloride and frozen at –20°C. The cell-pellet is strongly yellow because of the content of FMN in DHODC.

3.2. Extraction and Purification

All operations during purification are carried out at 4°C.

3.2.1. Extraction

1. Frozen cells from 2 L culture (circa 16 g) are resuspended in 80 mL of buffer A and disrupted by ultrasonic treatment.
2. Add $MgCl_2$ to a final concentration of 5 m*M,* and Triton X-100 to a final concentration of 0.1% to dissolve the membranes.
3. The extract is cleared by centrifugation in an SS-34 rotor (Sorvall) at 13,000 rpm (20,000*g*) for 1 h (*see* **Note 5**).

3.2.2. First Chromatography on DE-52

1. The clear yellow extract is pumped (flow 1 mL/min) onto a column of DE-52 (1.6 × 25 cm) equilibrated with buffer B. The enzyme binds in a narrow zone at the top of the column.
2. After application of the sample, the column is washed first with 50 mL of buffer B and then with 50 mL of buffer C.
3. The enzyme is eluted with a linear gradient (400 mL) from 0 to 0.25 *M* sodium chloride in buffer C, while 10 mL fractions are collected. The enzyme appears from the column with a peak around 0.15 *M* NaCl.

3.2.3. Hydrophobic Interaction Column Chromatography

1. The active, yellow fractions from the DE-52 column are pooled and solid ammonium sulfate is dissolved in the liquid at a final concentration of 1.1 *M*.
2. A turbidity that forms after the addition of ammonium sulfate is removed by centrifugation (10 min at 12,000*g*). The pellet is colorless.
3. The clear supernatant is pumped (flow 0.5 mL/min) onto a column of Phenyl Sepharose (1.6 × 20 cm) which has been equilibrated with buffer D containing 1.1 *M* ammonium sulfate. The enzyme binds in a highly concentrated zone at the top of the column.
4. After application of the sample, the column is washed with a linear gradient (160 mL) from 1.1 *M* to 0 *M* ammonium sulfate in buffer D followed by 100 mL of

buffer D. The washing removes a substantial amount of contaminating protein, but DHODC remains bound although it spreads a little on the column during the wash.
5. The enzyme is eluted as a sharp peak by pumping buffer D containing 1% Triton X-100 through the column. This high concentration Triton X-100 gradually replaces the enzyme from the column, and the column material changes appearance to a more white and nontransparent texture above moving yellow zone of DHODC (*see* **Note 6**).

3.2.4 Second DE-52 Column Chromatography

1. The pooled fractions from the Phenyl-Sepharose column are loaded on a second DE-52 column (1.6 × 25 mL) equilibrated with buffer D. The flow rate is 1 mL/min.
2. The column is washed thoroughly with about 300 mL of buffer D to remove all Triton X-100, which is monitored by the UV-light absorption at 280 nm.
3. The enzyme is eluted in a somewhat broad peak by a linear gradient (400 mL) from 0 to 0.3 *M* sodium chloride in buffer D. The chromatography on the DE-52 column in the absence of Triton X-100 results in a loss of about one-third of DHODC, which remains stuck at the top of the column even at very high concentrations of NaCl, but we have accepted this loss of enzyme in order to be able to replace Triton X-100 with other detergents (*see* **Note 7**).

3.2.5. Concentration and Storage

1. The active fractions from the second DE-52 column are pooled and concentrated using centrifugation spin columns (Amicon, Centriprep®).
2. For most purposes, the enzyme was dialyzed against buffer D containing 50% glycerol and stored in liquid form at −20°C at a concentration around 10 mg/mL.
3. Prior to crystallization the protein sample was dialyzed against a solution of 25 m*M* of sodium phosphate pH 7.0 containing 0.1 m*M* EDTA and 10% glycerol and stored in 0.5 mL aliquots at −20°C.

3.3. Crystallization

The crystallization of DHODC has been described previously by Rowland et al. ***(36)***. Crystals were obtained by the vapor diffusion technique using 5 μL sitting drops in microbridges placed over a 0.6-mL reservoir solution in the Linbro plates closed with cover slides. The experiments were carried out at room temperature. The drops were made from 2.5 μL protein solution (12–15 mg/mL DHODC) and 2.5 μL of the reservoir solution. Crystals could be obtained with reservoir solutions that have the following composition: 0.1 *M* sodium acetate, sodium formate in the concentration range 3.9–4.4 *M*, pH 4.0–5.5, and 25 m*M* β-n-octyl β-D-glucoside (β-OG). Prior to equilibration the drops had a composition contained 6.0–7.5 mg/mL of DHODC, 12.5 m*M* sodium phosphate pH 7, 0.05 m*M* EDTA, 5% glycerol (from the protein solution), 12.5 m*M* β-OG, 0.05 *M* sodium acetate, and 1.95–2.2 *M* sodium formate with a pH of 4.0–5.5, while

the reservoir solutions contained 0.1 *M* sodium acetate, and 3.9–4.4 *M* sodium formate with a pH of 4.0–5.5. With reservoir solutions in the afore mentioned range of sodium formate concentrations and pH, the enzyme crystallized within 1–2 wk as yellow needles of the approximate dimensions $1.5 \times 0.15 \times 0.15$ mm. The crystals have small whiskers at one end that was cut away to make the crystals suitable for X-ray diffraction experiments. To be able to measure diffraction data from crystals under cryogenic conditions, the crystals had to be soaked for a few seconds in a cryoprotecting reagent containing 4.5 *M* sodium formate, 0.1 *M* sodium acetate at the crystallization pH and 10% glycerol. The X-ray diffraction experiments showed that the crystals are tetragonal. To overcome the phase problem the selenomethionine substituted protein was prepared (*see* **Note 8**). It could be crystallized under the same conditions as the native enzyme. The structure determination was achieved by the MAD (multiple anomalous dispersion) method based on diffraction data collected with synchroton radiation at three different wavelength around the Se-absorption edge. Further details are described by Nørager et al. ***(28)***.

4. Notes

1. Buffers are prepared using doubly distilled water. They were prepared by dilution of five-times concentrated stock solutions and mixed with NaCl from a 5 *M* NaCl stock solution that was passed through a nitrocellulose filter to remove unwanted particles.
2. Other *E. coli* strains can be used, but the F' *proAB lacI^{q}ZΔM15 Tn10* episome, which directs the overproduction of the LacI repressor, is needed because the plasmid does not itself carry a *lacI* gene. The overproduction of all types of DHOD is toxic to *E. coli* and transformation with plasmid pAG1 is not possible unless the DHOD expression is kept repressed.
3. It is advisable to use freshly transformed cells and keep the culture exponentially growing until the final culture reaches stationary phase before harvest. If growth of culture is interrupted, it should preferably be done at a low cell density, e.g., at $OD_{436} \leq 0.5$. If the preculture has been grown into stationary phase, plasmid-free cells tend to outgrow the plasmid containing cells when the preculture is diluted into fresh medium, because the added ampicillin is rapidly broken down. This behavior is in all likelihood related to the fact that the copy number of plasmid pAG1 (and other relaxed plasmids), and hence the production of β-lactamase, increases dramatically when the culture approaches stationary phase. It is possible to store the transformed cells if an aliquot of the uninduced culture at a low cell density ($OD_{436} \leq 0.5$) is mixed with 20% glycerol and the frozen at –20°C. However, in that case, it is advisable to spread the cells to single colonies on an LB-agar plate with 0.1 mg/mL of ampicillin and test a few colonies for high-protein production in small cultures.
4. An "autoinduction" of *pyrD* expression from plasmid pAG1 occurs at a cell density about $OD_{436} = 2$. The reason is that the concentration of repressor binding sites on pAG1 in cultures approaching stationary phase exceeds the amount of LacI repressor produced from the stringently controlled F'-episome. The production of

DHODC from pAG1 is almost as high in "uninduced" stationary cultures as it is in cultures that are induced by addition of IPTG, but because this "autoinduction" may depend on subtle differences in the culture conditions, we have retained the induction with IPTG as described. The "autoinduction" of protein expression in stationary cultures, which we have seen with several plasmids where repression relies upon a *lacI* gene on an F'-episome, may also contribute to the strong tendency of plasmid-loss and low protein production in cultures that are inoculated with outgrown precultures.

5. When DHODC was purified from bacteria that expressed the protein either from the chromosomal *pyrD* gene or from low production plasmids ***(23)*** we disrupted the cells by use of a French press and isolated the membranes, which contained near 100% of the enzyme, by centrifugation. The protein was then released from the membranes by addition of Triton X-100 ***(37)***. The isolation of membranes prior to release of the enzyme gave a substantial purification (≥ 10-fold), but with the large overproduction of DHODC, achieved by the use of plasmid pAG1, the majority of DHODC remains in the supernatant, when the membranes are isolated. Therefore, this step is omitted from the purification procedure and the membranes are dissolved by addition of Triton X-100 prior to all fractionation.
6. The Phenyl-Sepharose column can be regenerated by extensive washing with 20% ethanol in water. The removal of Triton X-100 can be followed by monitoring the UV-absorbance.
7. The behavior of DHODC during chromatography on the DE-52 ion column in the absence of detergent is unusual. At low ionic strength, the enzyme appears to bind to the column material primarily by electrostatic forces and be released by a moderate salt concentrations. However, at high-salt concentrations, it sticks to the column material by hydrophobic interactions. In an attempt to elute the protein from the DE-52 column in a more-concentrated manner than obtained by the described salt gradient, we applied a solution of 1 *M* NaCl in buffer D to the column directly after Triton X-100 had been removed. All of the enzyme remained at the column during the high salt wash, and a part of it (about two-thirds) was eluted by a backward gradient from 1 *M* to 0 *M* NaCl in buffer D with a peak about 0.15 *M* NaCl.
8. Strain SØ6735 transformed with pAG1 was used to produce selenomethionine substituted DHODC for crystallization and structure determination ***(28)***. The strain was grown in the phosphate buffered minimal (A+B)-medium ***(35)*** supplemented with glucose (0.5%), methionine, leucine, isoleucine, and valine (all at a concentration of 50 mg/L) and with uracil (20 mg/L) and ampicillin (100 mg/L). Uracil was added because the *rph-1* mutation in strain DL41 (a derivative of MG1655) has a polar effect on transcription of the *pyrE* gene, which generates a strong stress in the supply of pyrimidine nucleotides and a reduced growth rate in pyrimidine free media ***(38)***. The preculture was grown in a medium supplied with normal L-methionine. At OD_{436} = 0.5 the preculture was cooled in an ice bath. The cells were harvested by centrifugation, washed with basal salt medium, and resuspended at an OD_{436} = 0.05 in 2 L of prewarmed medium, similar to the medium described aforementioned, but with DL-selenomethionine (0.1 g/L) replacing L-methionine.

After a few minutes, the growth rate declined to half of that seen in the preculture, indicating that all L-methionine had been consumed and that the cells were now thriving on selenomethionine. The synthesis of DHODC was induced at OD_{436} = 0.5 and the culture was left to reach stationary phase overnight at an OD_{436} of 2–3. Harvest of the cells, extraction, and protein purification was performed as aforementioned with the notable exception that 1 m*M* dithiothreitol (DTT) was included in all the buffers to prevent oxidation. Furthermore, only 0.9 *M* ammonium sulfate was added to the enzyme solution prior to application on the Phenyl-Sepharose column and the subsequent gradient changed accordingly to go from 0.9 *M* to 0 *M* ammonium sulfate. The reduction in the ammonium sulfate concentration was made because the selenomethionine substituted DHODC precipitates in the presence of 1.1 *M* ammonium sulfate. The yield of DHODC, fully substituted with selenomethionine, was circa 10 mg per liter of medium was.

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3

A General Approach for Heterologous Membrane Protein Expression in *Escherichia coli*

The Uncoupling Protein, UCP1, as an Example

Alison Z. Shaw and Bruno Miroux

1. Introduction

It is well accepted that one of the major limitations in membrane protein structure determination is to obtain enough protein material (for review, *see* **ref. *1***). A batch of approx 5 mg is necessary for the first round of crystallization screening, for example. Because many of the most interesting membrane proteins are often expressed at very low levels, it can be difficult, and perhaps not desirable, to purify them from their natural source; using an overexpression system is the obvious alternative. Owing to the great range of expression vectors available and the ease of use, *Escherichia coli* has proven to be the expression host of choice, particularly for small, cytoplasmic proteins. Heterologously expressed membrane proteins are often toxic to *E. coli*, which prevents cell growth and limits protein yields. In this chapter, we describe how an "in vitro evolution" approach can be used to produce *E. coli* strains, e.g., C41(DE3) and C43(DE3), which are better suited than BL21(DE3) to expression of some membrane proteins. Expression of the uncoupling protein (UCP1) in C41(DE3) is given as an example.

UCP1 plays a part in what is known as "proton leak." This curious phenomenon has been recognized for many years and is defined as the dissipation of the proton gradient existing across the inner mitochondrial membrane by routes other than through ATP synthase. The significance of this phenomenon is gradually coming to light through the study of the uncoupling protein family. UCP1, the most well-characterized member is known to provide this "unproductive" proton conductance pathway in brown adipose tissue (BAT) mitochondria and

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is activated during nonshivering thermogenesis, leading to the production of heat (for review, *see* **ref. *2***). The recent cloning of UCP2 and UCP3, both exhibiting high sequence identity with UCP1 has promoted interest in this field ***(3,4)***. They are expressed in tissues other than brown adipose tissue and provide potential therapeutic targets for the treatment of metabolic diseases such as diabetes and obesity and of inflammatory diseases ***(5–7)***.

Hydropathy analysis of these 33-kDa membrane proteins proposes six transmembrane domains, confirmed for UCP1 by antibody mapping which also reveals the location of N and C terminals in the intermembrane space ***(8)***. The organization of UCP1 into three repeats of approx 100 amino acids is apparent from the amino acid sequence, with each domain containing two transmembrane segments ***(9)***.

Both the full-length rat UCP1 and fragments of the protein (corresponding to each of the three repeats) have been expressed with histidine tags, in *E. coli* C41 (DE3) ***(10)*** as inclusion bodies. A method for the expression is described here, alongside a protocol to generate improved *E. coli* strains for membrane protein expression and some tips on finding the best conditions for maximizing protein yields.

2. Materials

1. 1X LB Medium: 10 g Bacto Tryptone (Difco #0123-17-3), 5 g Bacto Yeast Extract (Difco #0127-17-9), 5 g NaCl. Add sterile water to 1 L. Adjust the pH to 7.2–7.5 with NaOH (a few pellets of the solid) and autoclave.
2. 2X TY Medium: 16 g Bacto Tryptone (Difco #0123-17-3), 10 g Bacto Yeast Extract (Difco #0127-17-9), 5 g NaCl, 2 g glucose. Add sterile water to 1 L. Adjust the pH to 7.0 with NaOH (a few pellets of the solid) and autoclave.
3. IPTG (Isopropyl-beta-D-galactoside): A 1 M stock can be prepared in water and stored at −20°C. IPTG can be obtained from Sigma-Aldrich.
4. Antibiotics: Working concentrations of some antibiotics:

Antibiotic	(μg/mL)
ampicillin	100
carbenicillin	100
chloramphenicol	34
kanamycin	30
rifampicin	200
tetracycline	12.5

5. 5X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 4.0 mL distilled water, 1.0 mL 0.5 M Tris-HCl, 0.8 mL glycerol, 1.6 mL10% SDS, 0.4 mL β-mercaptoethanol, 0.2 mL 0.05% (w/v) bromophenol blue.
6. Inclusion body buffer: 300 m*M* NaCl, 10 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.5, 4 m*M* phenyl methyl sufonyl fluoride, (PMSF) (Sigma-Aldrich), added at the time of use. A 1 *M* stock of PMSF can be prepared in methanol and stored at −20°C. This protease inhibitor is highly toxic.

3. Methods

3.1. Designing Contructs for Expression

As well as making expression constructs of the full-length protein, it may make sense to break up your protein into smaller fragments. In that case, it is essential to think carefully about where to put the boundaries. Check hydropathy data and use programmes such as SMART (protein domain identification) and Jpred (secondary structure prediction) to identify potential domain boundaries (*see* **Note 1**). In general, it is best to avoid having hydrophobic residues on the ends of protein constructs because they may induce aggregation.

3.2. Choosing Expression Vectors

Having decided on which sequences to clone, the next step is to choose from the plethora of expression vectors. It is very difficult to know which vector will give the highest yields for the particular protein of interest and often the best strategy is to try a number of different vectors in parallel and screen for expression on a small scale (*see* **Subheading 3.4.**).

Optimal conditions are achieved when the plasmid remains stable throughout the growth of the culture and when expression of the protein is slighly toxic to the cell, but not so much that cell growth is seriously impaired. Unfortunately, expression systems are unpredictable because the strength of the promoter depends very much on the stability of the mRNA of the target gene ***(11)***. For instance, if the mRNA is very stable then a weak promoter will make a good balance to achieve a high level of expression of the target gene without toxicity to the host. On the other hand, if the mRNA of the target gene is highly unstable, then even the strongest *E. coli* promoter will be useless. Therefore, as a general rule, we advise you to clone your target gene in two to three very different expression vectors and to transform two to three different bacterial strains in order to cover weak, intermediate, and strong expression systems. In the case of the well-known T7 based RNA polymerase expression system ***(12)***, the low copy number pET vectors (Novagene) could be tested alongside the high copy number pRSET vector (Invitrogen) or the pMW7 vector ***(13)***. Other low or high copy number vectors with weak promoters should also be tested, such as the arabinose promoter or the lac promoter (*see* **Note 2**).

3.3. The Bacterial Host

When it comes to expressing the protein, the main difficulties are to balance the efficiency of protein production with the toxicity of expression; and to optimize yields of correctly folded protein without driving the equilibrium too far toward formation of inclusion bodies. This depends very much on the combination of vector and bacterial host. In this section we describe a general method

that allows you to assess (*see* **Subheading 3.3.1.**) and improve your expression system by selection of the bacterial host (*see* **Subheading 3.3.2.**).

3.3.1. Identifying a Suitable Host Strain

The method relies on the fact that the growth behaviour of your host strain on solid medium (LB-agar plates) will closely reflect its behaviour in liquid medium. For instance, if the bacterial host harboring the plasmid is unable to form a colony on an agar plate containing the expression inducer, then in liquid medium, cells may well start to die once expression is induced, and may lose the plasmid very rapidly. Eventually cells lacking the expression vector will overgrow the culture and very little heterologous protein will be obtained. This is what we have observed in experiments using the T7 expression system ***(10)***.

A strategy for identifying a suitable host is described in **Fig. 1**. It can be divided into several stages, the first of which is to check that your empty vector is not toxic to your bacterial host, especially if you use a T7 expression system ***(14)***. Cells harboring vectors that do not contain your inserted gene should form regular sized colonies on plates containing the inducer. If not, then you should either change the combination of vector and host or select mutant hosts by following the procedure described in **Subheading 3.3.2.** (*see* **Fig. 2**).

This first step is important so you can be sure that any potential toxicity comes as a result of the inserted gene and is not from the plasmid alone. You should now proceed with the vector containing the membrane protein gene and check the appearance of colonies on both LB-Agar plates (+antibiotic) and on plates also containing inducer. It is possible that only very small colonies form on the LB-agar plates, even without inducer (*see* **Note 3**). The only option in that case is to try a new host or a new vector.

Provided the colonies are normal sized in the absence of inducer, you should see how they look when inducer is added to the plates. As shown in **Fig. 1**, there are three possibilities at this stage. The first possible outcome is that the size of the colonies does not change at all with or without the inducer. This is a bad sign and probably indicates that the level of expression of your protein is very low or at least not optimal. Of course you can proceed and check the expression level of the target gene in liquid medium, but it is also wise to change to a stronger expression system.

The second possibility is that cells form smaller colonies in the presence of the inducer. This is a good sign but does not always guarantee that expression will be successful (*see* **Note 4**). In the case of the T7 expression system this observation has been associated in many cases with a high level of expression of the target gene ***(15)***, which could still be optimised by changing the growth conditions (*see* **Subheading 3.4**).

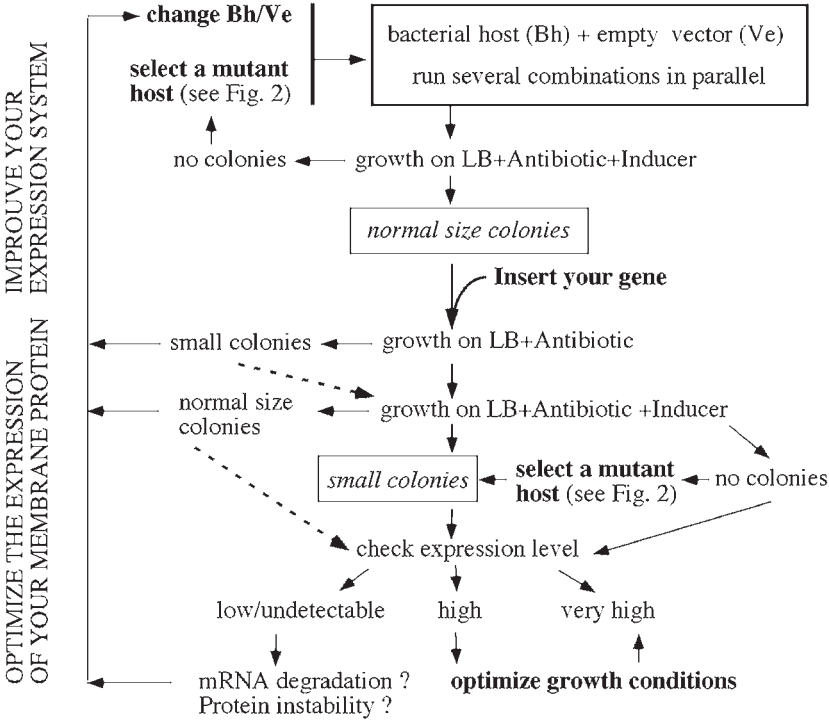

Fig. 1. General strategy for choosing the best combination of expression vector and bacterial host. The strategy relies on the fact that the expression system by itself should not be toxic and that the expression of the target gene is optimal when the growth of the cells after induction is only slightly reduced. This is reflected on agar plates containing the inducer by the formation of small size colonies.

In the third case, cells are unable to form colonies in presence of the inducer. This is very typical for membrane proteins but does not necessarily mean that the target gene is toxic. Our experience with the expression of the mitochondrial carriers and of the F1Fo ATP synthase is that some optimization is necessary to identify the right timing of induction or the appropriate strength of the promoter (*see* **Note 5**). Opting for host selection would be a sensible choice at this stage.

3.3.2. Obtaining Mutant Strains by Host Selection

A procedure for selecting a bacterial strain, which is better suited to expressing your target gene is illustrated in **Fig. 2**. In this example, the starter strain is BL21(DE3) and the expression plasmid, pGFP encodes the green fluorescent protein from *Aequora Victoria* ***(16)***. The steps are as follows:

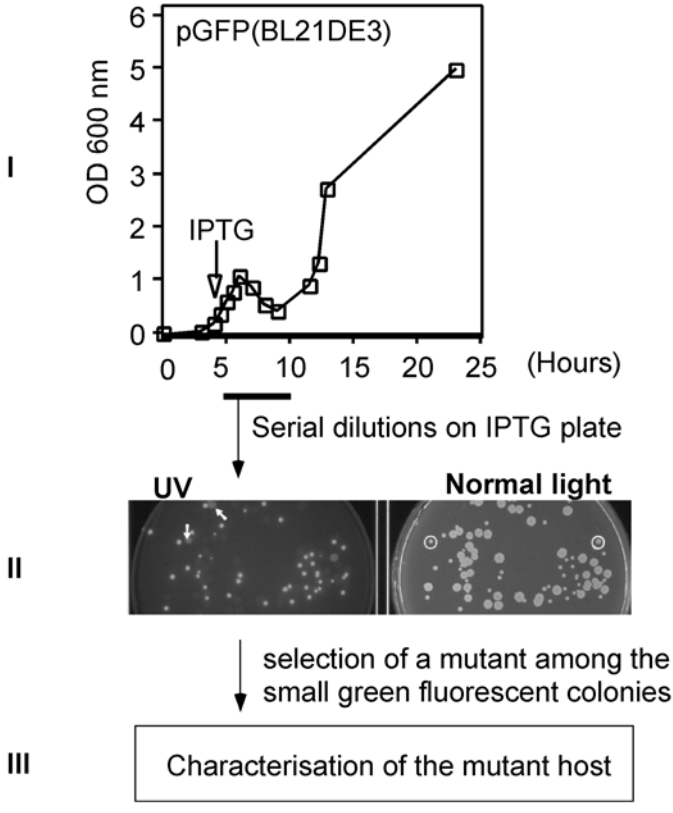

Fig. 2. Selection of mutant of *E. coli* BL21(DE3) using GFP as a gene reporter.

The green fluorescent protein is expressed in BL21(DE3). Three hours after induction of its expression, cells were diluted and plated out onto agar medium containing IPTG and ampicillin. The following morning, the plates were illuminated either with a UV lamp or with a normal lamp. Mutants that have kept the ability to express GFP form small fluorescent colonies while normal size bacterial colonies do not express GFP. White arrows and circles indicate the exceptions to rule.

1. Use a fresh colony to inoculate 50 mL of LB medium and induce expression (in this case, by adding IPTG to 0.7 m*M* final concentration). Monitor cell growth by measuring the optical density (OD) at 600 nm.
2. Typically, the OD decreases slightly after induction because some of the bacteria have immediately lysed. Cell growth does not recover until 1–3 h after the inducer is added. At the point just before the OD starts to increase again, cells should be diluted with LB medium or sterile water to make serial dilutions between 1:10 and 1:10,000. Cells are then immediately plated out on to LB-agar plates containing the appropriate inducer (e.g., 0.7 m*M* IPTG and antibiotic). Because the frequency of

viable cells and of mutants is variable, it is critical to make progressive dilutions of the culture in order to isolate individual colonies.

3. After overnight incubation at 37°C, two populations of cells should appear and in the case of GFP, the interesting mutants are immediately revealed under UV light. As illustrated in **Fig. 2**, most of the normal-sized colonies are not fluorescent. They represent mutants that have lost the ability to express GFP but keep ampicillin resistance. In some rare cases, some of these mutants like those indicated by a white arrow in **Fig. 2**, are poorly fluorescent. In contrast, almost all small colonies (except two of them highlighted by a white circle) are highly fluorescent, indicating high GFP expression. These are the mutant hosts to be isolated.
4. In order to have a strain you can work with, the cells must be cured of the plasmid. To do this, it is better to avoid the use of mutagenic compounds such as acridine orange; the simplest method consists of maintaining the selected strain in exponential phase for a week in LB medium without antibiotic or IPTG. Each day, you should make a serial dilution of the culture and plate out the cells on LB-agar plates containing IPTG but without antibiotic. After selection, the size of the colonies on IPTG plate can be considered as a signature of the mutant. On IPTG plates, mutant cells that have lost the plasmid will form normal size colonies and in the case of the GFP mutant, they will, of course, have lost their green fluorescence. To check that the mutation is in the bacterial host and not in the plasmid it is important to retransform the original plasmid into the isolated host and verify that the "colony size phenotype" on an IPTG plate is conserved.

3.4. Optimization of Growth Conditions

Optimization of expression conditions is essential for proteins that are difficult to express in high quantities and this can be done quite easily on a small scale. Temperature, type of medium, concentration of inducer, timing / length of induction, and degree of aeration can all influence cell growth and the yield of protein. The efficiency of aeration depends very much on the type of vessel used to grow the bacteria in, the speed of rotation, and the volume of culture. It is difficult and, therefore, not useful to test the influence of that factor in a small-scale screen. However, temperature, IPTG concentration, timing and length of induction can all be easily tested provided you have an Eppendorf thermomixer, or similar shaking / heating block.

A simple screen for growth conditions can be performed with 200-μL cultures, set up in 2-mL Eppendorf tubes. The number of tubes you prepare will depend on how many conditions you want to test at one time and how many different constructs you have. Eppendorf thermomixers contain 24 spaces, so 24 conditions at one temperature is the maximum you could try. If you have more than one shaker, it is convenient to test several temperatures in parallel.

A manageable setup is a total of 24 cultures split between two temperatures

Table 1
Optimization of Growth Conditions of the *E. coli* Culture

Temperature of induction: 37°C or 15°C		
Construct A (IPTG)	Construct B (IPTG)	
0.2 m*M*	0.2 m*M*	Induce at
0.7 m*M*	0.7 m*M*	OD_{600} = 0.6
1 m*M*	1 m*M*	
0.2 m*M*	0.2 m*M*	Induce at
0.7 m*M*	0.7 m*M*	OD_{600} = 1
1 m*M*	1 m*M*	

(room temperature [RT] and 37°C or RT and 15°C, for example). Two different constructs can be tested for a range of inducer concentrations and also timing and length of induction. A sensible range of conditions is illustrated in **Table 1**. The two constructs tested simultaneously are called A and B; 0.2 m*M*, 0.7 m*M*, and 1.0 m*M* refer to IPTG concentrations (*see* **Table 1**).

1. Prepare 5 mL master cultures of A and B by inoculating LB medium + antibiotic with a single colony from a fresh transformation (*see* **Note 6**). Incubate at 37°C and monitor cell growth by measuring the $OD_{600nm.}$
2. When the OD approaches 0.6, distribute 200-µL of each culture into 2-mL Eppendorf tubes; three for each construct in both the 37°C shaker and the 15°C shaker makes a total of 12 tubes. Induce expression by adding the appropriate volume of 100 m*M* IPTG.
3. Continue to measure the OD_{600} of the master cultures and repeat the previous steps once the optical density approaches 1.0 (*see* **Table 1**). Keep a sample of the uninduced master cultures. These will serve as controls for the SDS-PAGE to help you detect which band corresponds to the overexpressed protein.
4. Take 10-µL samples of the induced cultures after 3 h, 6 h and after overnight incubation.
5. Mix with 5X SDS-PAGE buffer and boil for 10 min to lyse the cells and denature the samples.
6. Spin them on a bench-top centrifuge for 10 min at 14,000*g* before analysing the supernatant on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) together with the control "uninduced" samples. You should end up with a total of 72 samples +2 controls. That is a rather colossal number if you want to run SDS-PAGE minigels so cut them down by being selective over the "induction time" samples. Only run the 3-h samples of the cultures grown at 37°C, run just the 6-h samples of those at grown at RT and take the overnight samples if the induction temperature was 15°C (*see* **Note 7**).

3.5. Large-Scale Expression and Harvesting of Cells

Once the optimum growth conditions have been identified in the small-scale screen, protein can be expressed in larger volumes. The level of expression will determine what volume of culture you should grow. It is wise to start with 1 or 2 L and perhaps grow two different constructs in parallel and then scale up later if necessary.

1. Make a 50-mL starter culture in a sterile 250-mL conical flask by inoculating medium + antibiotic with cells from a single colony. Grow overnight at 37°C with shaking.
2. The following morning prepare a chosen number of 2-L Erlenmeyer flasks with 500 mL of medium + antibiotic each. Prewarming the medium to 37°C will speed up the cell growth, but is not essential.
3. Inoculate each 500 mL with 5 mL of starter culture.
4. Grow the cells at 37°C with shaking at about 150–200 rotations per min. Monitor cell growth by measuring the OD_{600} every 30 min.
5. When the induction time is up, harvest the cells by centrifuging at 2000*g* for 15 min at 4°C (*see* **Note 8**).

3.6. Expression Conditions for UCP1

It has not been possible to find conditions where the full-length UCP1 protein can be both highly expressed and correctly inserted into the plasma membrane; in C41(DE3) the recombinant protein accumulates in high amounts as inclusion bodies instead. Our strategy for obtaining pure, functional protein has been to denature and refold these inclusion bodies. However, there are a number of difficulties associated with refolding of membrane proteins. The absence of a native membrane to insert into is a potential problem and the chances of misfolding are certainly likely to increase according to the size of the protein and number of transmembrane domains. Our logic was to isolate each of the three repeats of UCP1 as individual constructs. With each one possessing two transmembrane spans and a 40-residue loop, the chances of correct refolding are higher than for the full-length protein.

The full-length UCP1 and the fragments of the protein were cloned into a pET like vector and a T7 based high copy number plasmid (gift of M. Runswick) derived from pMW7 ***(13)***, which both give protein with a 6X histidine tag. Depending on the vector, the tag is either at the N-terminus and separated from the protein by a Tev (Tobabcco Etch Virus) protease cleavage site, or is C-terminal and not cleavable. As a selectable marker, both vectors confer ampicillin resistance and a T7-*lac* promoter initiates transcription in the presence of IPTG.

1. C41(DE3) cells that harbor the expression plasmid are grown at 37°C in LB medium until the OD_{600} reaches 0.6.

2. Overexpression is induced by adding IPTG to a final concentration of 0.7 m*M*. Protein yields are equally good whether cells are induced at 37°C for 3 h or at 25°C overnight.
3. Cells are harvested by centrifugation and lysed at 4°C by passing them twice through a French pressure cell. Unbroken cells are isolated by spinning the lysate at 600*g* for 15 min.
4. The supernatant is recentrifugated at 10,000*g* for 20 min to isolate the inclusion bodies.
5. A homogeniser is used to resuspend the inclusion bodies in Inclusion body buffer to a concentration of 30 mg total protein per mL. Comprising mostly overexpressed protein (*see* **Fig. 3**), the yield of inclusion bodies is typically 250–300 mg L of culture.

Notes

1. The following Web resources are useful in planning and implementing the expression of membrane proteins:
   SMART (protein domain database)
   http://smart.embl-heidelberg.de/
   Jpred (secondary structure prediction)
   http://jura.ebi.ac.uk:8888/
   EMBL Protein Purification and Expression Unit
   http://www.embl-heidelberg.de/ExternalInfo/geerlof/draft_frames/frames_which_vector_ext.htm/
   ExPasy
   http://www.expasy.ch/
2. With such a large variety of vectors to test out, it is not difficult to get overloaded so keep the number of constructs well within a limit you can realistically manage and keep the cloning steps as simple as possible (try to use the same restriction sites for all the constructs, for example). For more details on the types of vectors available, the web page of the EMBL Protein Purification and Expression Unit is a good resource. *http://www.emblheidelberg.de/ExternalInfo/geerlof/draft_frames/frame_which_vector_ext.htm/*
3. We have frequently observed that some expression systems are so leaky (i.e., overexpression occurs before inducer is added) that even in the absence of inducer on the plates, cells are unable to form regular sized colonies. In severe cases, such as the mitochondrial ADP/ATP carrier or the B-subunit of the *E. coli* ATP synthase expressed in BL21(DE3) with the pMW7 vector, cells form pinhead sized colonies which do not grow in liquid medium ***(15)***. In such cases, a new combination of vector and host needs to be found.
4. If in spite of attempts to optimize growth conditions, the expression level is still very low you should consider investigating the stability of both the mRNA and the target protein. Predictive programmes allow the determination of secondary structure susceptible to RNA degradation ***(11)*** and of the half-life of your protein in various expression systems (*see* ExPasy web site). Silent point mutations may help in

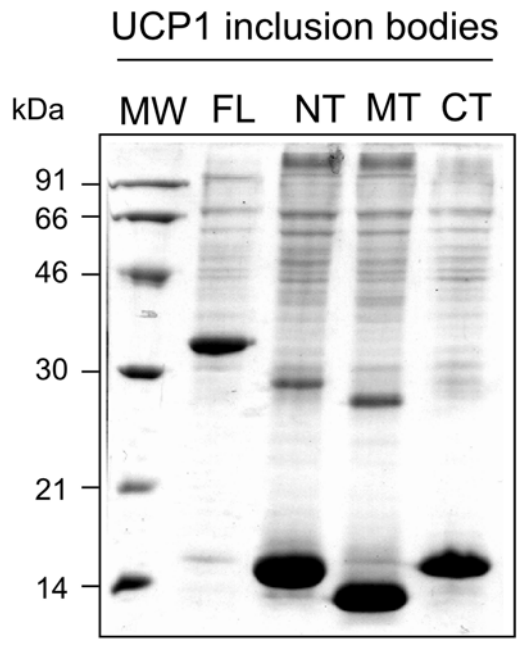

Fig. 3. Analysis by SDS-PAGE of the inclusion bodies preparation of four different constructs of rat UCP1: the full length UCP1 (FL), its N-terminal third (NT), middle third (MT), and C-terminal third (CT). Ten micrograms of inclusion bodies were loaded on the gel. The gel was stained using Coomassie blue dye.

stabilizing mRNA and using a protein fusion tag (e.g., Glutathione S transferase, GST) has also been known to increase protein stability.

5. Obtaining stable, correctly folded protein does not stop at translation, membrane insertion is necessary and the cell may need time for metabolic adaptation such as lipid synthesis and membrane proliferation. Expression of the b-subunit of the *E. coli* ATP synthase is a very interesting example. The BL21(DE3) strain was intolerant to the pMW7(Ecb) plasmid even before induction of expression. Equally, expression in C41(DE3) was toxic to the cell and the b subunit was somehow misfolded. A specific mutant host, C43(DE3) was then selected as described in **Subheading 3.3.2.** and expression gave no signs of toxicity. Upon induction of expression, the bacterial host produced an internal membrane network in which the b-subunit was concentrated ***(17)***. This is why we strongly recommend selecting mutant hosts in order to specifically optimise the expression system for your membrane protein.
6. When inoculating a starter culture for expression, it is wise to start from a fresh colony rather than a liquid culture. There is a possibility that the cells may lose the plasmid or some recombination may occur. Ideally, you should start from a plate of freshly transformed cells.

7. It is important to bear in mind that good expression does not necessarily mean the final yield of purified protein will be high enough. It is worth extending the screen and testing for purification and cleavage (in case the protein has a tag or fusion protein) before deciding which vector is best. It is not uncommon that a tagged protein can be purified in high amounts but precipitates as soon as it is cleaved from the fusion partner!
8. When growing large cultures, if you want to induce the overexpression at a temperature lower than 37°C, it is important to lower the temperature of the incubator well in advance (1 h–30 mins) of the point at which you want to add the inducer. The incubator may cool down quickly, but the cultures will take some time to reduce in temperature. If you overshoot the point at which you should start cooling the incubator, put the flasks at 4°C until they reach the correct temperature.

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4

Expression of Membrane-Bound Iron–Sulfur Proteins

Dirk Schneider, Christian L. Schmidt, and Andreas Seidler

1. Introduction

Overexpression of membrane-bound proteins to obtain milligram quantities for biochemical and biophysical studies is usually a difficult task, especially for those proteins that span the membrane several times ***(3)***. An additional difficulty arises when these proteins need to incorporate organic or inorganic cofactors. In order to obtain a functional protein, this requires a host that is able to produce this cofactor or the cofactor has to be added to the growth medium and subsequently taken up by the host cells. Alternatively, the protein can be expressed as inclusion bodies and then refolded in the presence of the cofactor(s), a strategy successfully applied for bacteriorhodopsin and the light-harvesting complex of higher plants ***(3)***.

There are few examples of successful overexpression of membrane-bound FeS proteins; most of them are *Escherichia coli* (*E. coli*) proteins overproduced in *E. coli*. In fact, these proteins were globular subunits of large membrane-associated complexes like NADH dehydrogenase or fumarate ***(2)*** reductase ***(19)***.

The Rieske FeS protein is a membrane-associated compound of the electron transport chain of photosynthesis and respiration ***(4)***. It is a subunit of the cytochrome *bc* complex in mitochondria, chloroplasts, and bacteria. The protein consists of an N-terminal transmembrane helix and a globular domain located on the P-side (intermembrane space/thylakoid lumen/periplasm) of the membrane carrying a 2Fe2S cluster. In addition, this protein contains a disulfide bridge, which is known to be important for the stability of the FeS cluster. Because of the important role of this protein in electron transfer ***(5)***, it has been studied intensively in recent years. Successful overexpression of the Rieske protein with incorporated FeS cluster was reported only for those proteins

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derived from thermophilic *archea* ***(5,12)*** but not for those from mesophilic organisms. However, a truncated version of this protein from a mesophilic cyanobacterium was heterologously expressed and a significant fraction was found to carry a 2Fe2S cluster ***(6)***. In this chapter, we describe methods used for overexpression of three full-length Rieske FeS proteins from the cyanobacterium *Synechocystis* PCC 6803. Two of them, PetC1 (product of ORF sll1316) and PetC2 (product of ORF slr1185), are rather similar to the Rieske protein found in cytochrome b_6f complexes, whereas the other (PetC3, product of sll1182) is quite different in both sequence and size. The PetC1 protein had to be refolded and the iron–sulfur cluster had to be reconstituted in vitro after purification of the protein ***(13)***, the two other proteins were also obtained in a membrane-bound form carrying the 2Fe2S cluster (*see also* **ref.** ***14)***.

2. Materials

1. pRSET6a expression vector, pLysE plasmid (Novagene).
2. *E. coli* strains DH5α, BL21(DE3).
3. Oligonucleotide primers for polymerase chain reaction (PCR).
4. Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase.
5. Plasmid miniprep kit, DNA purification kit (for purification of DNA from agarose gels).
6. LB medium containing 100 μg/mL ampicillin with or without 50 μg/mL chloramphenicol.
7. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
8. PBS buffer: 137 m*M* NaCl, 2.7 m*M* KCl, 12.8 m*M* Na_2HPO_4, 1.76 m*M* KH_2PO_4, pH 7.2.
9. Sonicator.
10. Proteinase inhibitors:phenylmethylsulfonyl fluoride (PMSF), p-tosyl-L-lysine chloromethyl ketone (TLCK).
11. Magnesium chloride.
12. DNase I (Boehringer).
13. 50 m*M* Tris-HCl, pH 8.3.
14. Wash buffer: Phosphate-buffered saline (PBS) containing 25% sucrose, 1% Triton-X-100, 5 m*M* ethylene diamine tetraacetic acid (EDTA).
15. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.

3. Methods

The following paragraphs describe

1. The construction of expression plasmids for the overexpression of three different *Synechocystis* Rieske proteins;
2. Conditions for successful protein expression; and
3. Characterization of the expressed proteins.

3.1. Expression Vector

In general, the conditions for successful overexpression of membrane proteins cannot be predicted and must be determined empirically. Many membrane proteins seem to behave differently and the expression levels depend on many factors as discussed in ***(3)***. For the expression of proteins in *E. coli*, many different plasmids and promoter systems have become available in the recent years. The different features of these systems strongly influence the expression of foreign proteins in *E. coli*. Therefore, each system has to be optimized either to obtain a functional membrane protein incorporated into *E. coli* membranes or to produce large quantities of insoluble protein, localized in inclusion bodies.

In recent years, it turned out that the integration of proteins into the *E. coli* membrane is an important and rate-limiting factor ***(1–3)***. In the case of polytopic membrane proteins, the use of a low-copy-number plasmid in combination with a weak promoter may be most promising, because low-level bacterial expression could result in a higher degree of integration of the protein into the cytoplasmic membrane of *E. coli* ***(3)***. However, in our hands, the use of the strong ψ10 promoter of the bacteriophage T7 for the Rieske FeS protein was most successful.

3.1.1. pRSET6a

The pRSET family of expression plasmids are T7 promoter-based expression vectors derived from pBluescript ***(15)***. They are high-copy-number vectors featuring translational start and stop elements and a multiple cloning site. Because of the T7 promoter, which is not recognized by the *E. coli* RNA polymerase, the system is "off" in *E. coli* strains not carrying the T7 RNA polymerase gene. In strains bearing this gene (like BL21 [DE3]) the T7 RNA polymerase gene is under the control of the *lacUV5* promoter and after promoter induction, proteins are usually produced at a very high level. In the uninduced state, the gene of interest is transcribed at a low level, even without additional induction of the promoter. This allows a moderate level of protein expression without significant inhibition of cell growth. The coexpression of T7 lysozyme from the plasmids pLysE or pLysS allows more stringent control of protein expression, because lysozyme is a natural inhibitor of the T7 RNA polymerase ***(17)***.

The described characteristics make the pRSET vectors a suitable system for the large scale production of proteins (after induction with IPTG) and for low-level expression (without induction), which can result in the functional expression and folding of a membrane protein and its incorporation into the *E. coli* cell membrane.

3.1.2. Preparation of Genomic DNA From Synechocystis PCC6803

Usually, the purification of genomic DNA from the cyanobacterium *Syechocystis* involves several steps. Briefly, after cell disruption the DNA is extracted by phenol/chloroform and afterward further purified. For the purpose of performing PCR, the DNA preparation does not have to be very pure, and quicker methods are effective. In ***(8)***, the authors describe a relatively fast method for the small-scale isolation of genomic DNA for PCR applications. Trying to use the simplest approach, we harvested 1.5 mL of cyanobacterial cells (*see* **Note 1**) by centrifugation, resuspended the cells in 100 μL TE buffer ***(11)***, and incubated the cells for 2–3 min at 100°C. Afterwards, the cells were sedimented again and the supernatant was transferred to a new reaction tube. 5–10 μL of this solution were used as DNA template in PCRs.

3.1.3. DNA Cloning

Molecular cloning was carried out using standard techniques described in ***(8)***. The three full-length *Synechocystis petC* coding regions were amplified individually by PCR. For cloning purposes, an *NdeI* site was introduced at the 5' end of the ORF including the translation start codon ATG, and a *BglII* or a *BamHI* site was introduced at the 3' end of the ORFs. The PCR-amplified DNA fragments were cleaved with *Nde*I and *BglII* or *BamHI*, respectively, and cloned into the appropriate restricted expression plasmid. After transformation of the plasmids into *E. coli* DH5α, the cells were grown overnight on standard LB plates containing 100 μg ampicillin/mL. Single colonies were tested afterwards for the insertion of the *petC* coding regions into pRSET6a. In order to exclude any error introduced by the PCR, the insert of one clone of each expression plasmid was finally sequenced.

3.2. Protein Expression

The level of expression, the localization of a membrane protein in *E. coli* (soluble, inclusion bodies, membrane), and its structure (folded vs unfolded) depends on many factors. It should be noted that the optimal expression conditions have to be determined for each individual protein by testing a number of important parameters like the *E. coli* strain, the growth medium, the expression temperature, the expression time, the addition of supplements, and so on.

3.2.1. E. coli Strain, Transformation, and Induction

Because the gene of interest in the pRSET plasmid is under the control of the T7 promoter, a strain carrying the λDE3 lysogen has to be chosen for protein expression. One of the most commonly used strains is *E. coli* BL21 (DE3), which was also used in this study (*see* **Note 2**). The cells were transformed with

the individual expression plasmids by standard techniques ***(11)***, plated on LB plates that contain the appropriate antibiotics, and incubated overnight at 37°C.

3.2.2. Expression of PetC1

1. Inoculate 10 mL LB medium containing 100 μg/mL ampicillin with a fresh single colony and shake the culture for 6 h at 37°C.
2. Centrifuge this culture to sediment the cells at 3000*g* for 10 min.
3. Resuspend the cells in 500 mL fresh medium.
4. Incubate the culture for 15 at 37°C with continuous shaking without addition of IPTG.
5. Harvest the cells for further protein purification and characterization.

3.2.3. Expression of PetC2 and PetC3 (see ***Note 2***)

1. Select a fresh single colony and inoculate 10 mL LB medium containing the appropriate antibiotics (100 μg/mL ampicillin plus or minus 50 μg/mL chloramphenicol).
2. Let the cells grow for 6–8 h at 37°C.
3. Sediment cells by centrifugation for 10 min at 3000*g* and resuspend the sediment in 5mL fresh medium containing the appropriate antibiotics.
4. Store cells overnight at 4°C.
5. The next morning, inoculate 500 mL fresh medium with 5 mL of cells (*see* **step 4**) and shake at 37°C.
6. At an OD_{600} = 0.6, induce protein expression by addition of 0.5 m*M* IPTG from a freshly prepared stock solution.
7. Harvest cells after 3–4 h for protein purification.

3.2.4. Cell Harvest

1. After protein expression, harvest cells by centrifugation (5000*g*, 10 min) and resuspend the cells in 20 mL PBS buffer.
2. The cells can be frozen at −20°C at this step without any degradation of the overexpressed proteins. Because of the freezing step, some cells are already disrupted and DNA is released. This can result in a slimy, tacky pellet if the cells were not resuspended before. Therefore, we recommend resuspending the cells before freezing.

3.3. Protein Extraction

3.3.1. Cell Disruption

1. Add 1 m*M* PMSF and 0.1 m*M* TLCK to the resuspended expression cultures.
2. Break the cells by sonication (*see* **Note 3**).
3. Add 25 m*M* $MgCl_2$ and 0.1 mg/mL DNase. Incubate the broken cells for 30 min at 37°C (*see* **Note 4**).

3.3.2. Fractionation of E. coli Cells

Possible strategies for the purification of the individual Rieske proteins from *E. coli* after overexpression are shown in **Fig. 1**. The strategy of choice depends

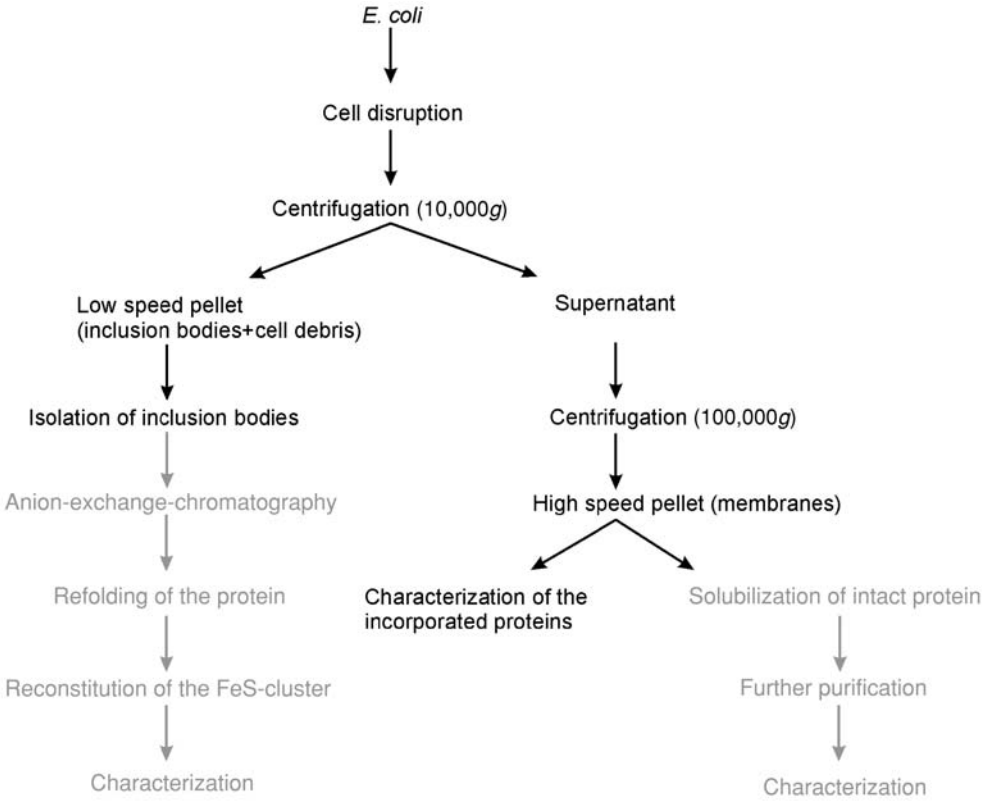

Fig. 1. Flowchart of the procedure used to purify the Rieske proteins overexpressed in *E. coli*. Black steps are described in detail in the text. Gray steps indicate possible further steps not described in this chapter.

on the localization of the expressed protein and the structure of the protein (native vs inclusion body).

3.3.2.1. Inclusion Body Isolation

1. Centrifuge the broken cells at 10,000*g* for 10 min.
2. Save the supernatant for the membrane preparation (*see* **Subheading 3.3.2.2.**).
3. Resuspend the sediment (inclusion bodies and cell debris) in 10 mL wash buffer with 1m*M* PMSF and 0.1 m*M* TLCK.
4. Centrifuge for 10 min at 10,000*g* and discard supernatant.
5. Repeat **steps 3** and **4** twice.
6. Resuspend the sediment in 20 mL water and centrifuge as in **step 4**. Repeat this step once.

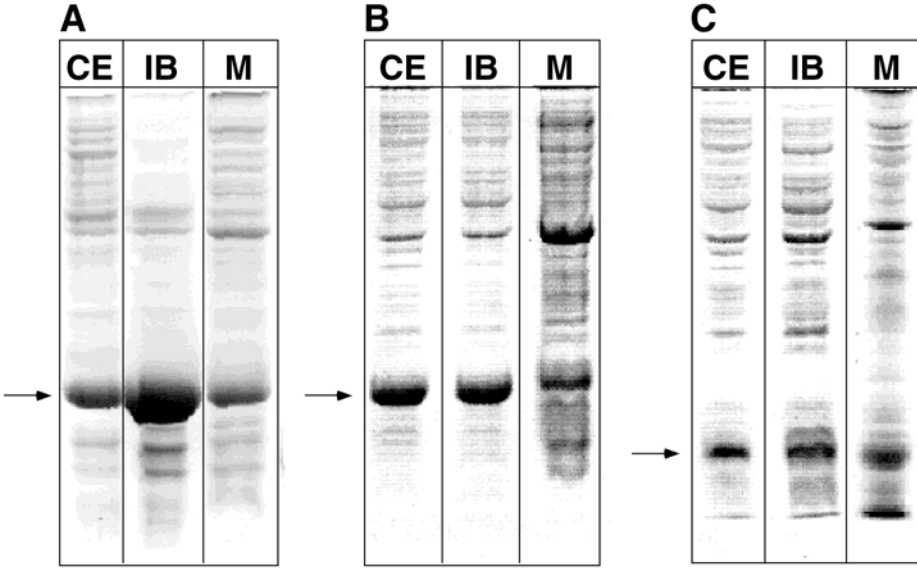

Fig. 2. Localization of the overexpressed Rieske proteins PetC1 **(A)**, PetC2 **(B)**, and PetC3 **(C)** in *E. coli.* CE, cell extract; IB, inclusion bodies; M, membranes.

7. Resuspend the pellet in 1 mL PBS buffer with 1 m*M* PMSF and 0.1 m*M* TLCK (*see* **Note 5**).
8. The purified inclusion bodies can be stored for further purification at −20°C (*see* **Note 6**).

3.3.2.2. Membranes

1. Centrifuge the supernatant from **step 2** (*see* **Subheading 3.3.2.1.**) in an ultracentrifuge for 30 min at 100,000*g*.
2. Resuspend the sediment in 500 μL 50 m*M* Tris-HCl, pH 8.3, 1 m*M* PMSF, 0.1 m*M* TLCK.
3. This membrane fraction can be stored at −20°C.

3.4. Characterization

The success of protein overexpression and purification can be easily determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The described method leads to a highly enriched Rieske protein (*see* **Fig. 2**); in the case of the *Synechocystis* PetC3 protein no additional purification step is necessary after isolation of the inclusion bodies (*see* **step 3.3.2.2.** and **Note 5**).

3.4.1. Rieske-Type EPR Signals

The Rieske protein was originally characterized by Rieske et al. ***(19)*** by the existence of the typical EPR signal of the iron–sulfur cluster. To check if the individual *E. coli* fractions (membranes, inclusion bodies) show a new EPR signal typical for the Rieske iron–sulfur cluster EPR spectra were recorded on a Bruker EPR200 spectrometer. The sample temperature was kept at 15 K by a helium cryostat. Typical parameters for a measurement were: 6.3 mW microwave power, 10 G modulation amplitude, and 9.44 GHz microwave frequency. The samples were completely reduced by the addition of a few grains of $Na_2S_2O_4$, partly reduced by addition of 5 m*M* sodium ascorbate, and completely oxidized by addition of 5 m*M* CeO_2 (*see* **Note 7**). For the first measurements, the protein samples were measured undiluted.

By this method it could be shown that in *E. coli* membranes of the strains overexpressing the *Synechocystis* PetC2 and PetC3 proteins, the typical Rieske EPR signal can be observed indicating the presence of a correctly folded protein in the membrane with an incorporated cluster (*see* **Fig. 3**). Whereas the iron–sulfer clusters of both PetC2 and PetC3 were completely reduced after addition of $Na_2S_2O_4$, the PetC3 EPR signal was almost not observable after partial reduction with ascorbate in contrast to PetC2. Under oxidizing conditions, a Rieske EPR signal was observed in none of the samples (*see* **Note 8**).

In the case of the PetC1 protein, a fraction of the protein is incorporated in *E. coli* membranes, but no typical EPR signal was detected. In order to obtain a folded protein with incorporated iron–sulfur cluster, this protein has to be folded and reconstituted in vitro ***(6,13)***.

4. Notes

1. The cultivation medium and conditions are described in ***(10)***.
2. As mentioned in **Subheading 3.1.**, the pRSET vector has some background expression even without extra induction of the expression by IPTG. Although this can be used for low-level expression of proteins (like described here for PetC1), this can also lead to cell death of the transformants if the protein is very toxic for *E. coli*. In the case of the *petC3* gene, no protein expression was achieved using BL21(DE3). Even the cotransformation of the helper plasmid pLysS, from which lysozyme is constitutively expressed at a certain level, is not sufficient to block the protein expression strongly enough. PetC3 can only be overexpressed successfully in a BL21(DE3) strain harboring the plasmid pLysE.
3. Alternative methods can be utilized for cell lysis including the use of a French press, treatment with lysozyme, or several cycles of freezing and thawing. BL21(DE3) cells harboring the pLysS or pLysE plasmids can easily be lysed by freezing once and incubation of the cells afterward at 37°C. The freezing step

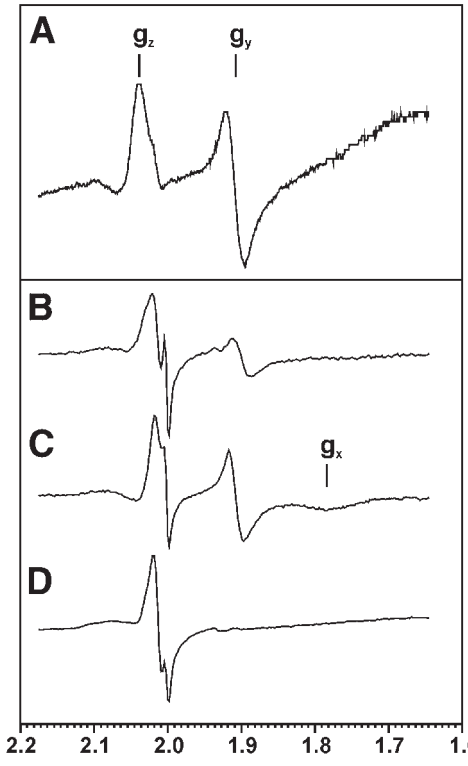

Fig. 3. EPR spectra form *E. coli* cell membranes containing PetC2 **(B)** and PetC3 **(C). (A)** Shows the EPR signal from the isolated and in vitro reconstituted PetC1 protein ***(13)***, and in **(D)** a control of *E. coli* cell membranes is shown. The positions of the $g_{y,z}$- values are indicated in **(A)**. The g_x-value could only be determined accurately for the PetC3 protein **(C)**.

In all membrane protein preparations an additional EPR signal with a g-value of 2.02 was detected, most likely caused by the presence of small amounts of copper. Beside this signal additional signals, typical for the presence of Rieske iron–sulfur clusters were detected. The samples were completely reduced by addition of $Na_2S_2O_4$. The protein concentration was 40 mg/mL.

breaks some cells, which leads to the release of internal lysozyme produced by the *lys* gene. The addition of 25m*M* $MgCl_2$ is recommended.

4. The released nucleic acids often result in the formation of a slimy, sticky pellet. The further fractionation of this pellet (cell debris, inclusion bodies, and membranes) can be more problematic under such conditions. The treatment of the sediment with DNase (and RNase) can improve further purification steps.

5. Although the overexpressed protein is highly enriched in inclusion bodies, usually the protein is not completely pure and further purification steps are required. Because PetC3 is virtually insoluble at this pH, the pellet can further treated with 50 m*M* phosphate buffer (pH 12.0). By this step, almost all of the *E. coli* proteins are solubilized, whereas PetC3 can still be sedimented by centrifugation. This additional washing step results in an electrophoretically pure PetC3 protein without further purification.
6. For further purification of the protein, we solubilize the final inclusion body pellet in 50 m*M* Tris-HCl pH 8.3 + 8 *M* urea and filtered this protein solution through a 0.45 μ*M* filter. After the isolation of the inclusion bodies, the protein is already highly enriched (*see* **Fig. 2**). In the case of the PetC3 protein, the inclusion body fraction is already electrophoretically pure. The proteins PetC1 and PetC2 can be further purified by one anion-exchange chromatography step and refolded and reconstituted in vitro. Because these steps are beyond the scope of this article, the interested reader is referred to ***(6,14)***.
7. Usually, $K_3Fe_2(CN)_6$ is used for the oxidation of Rieske proteins. Because the iron shows a very high electron paramagnetic resonance (EPR) signal, the usage of CeO_2 is recommended.
8. The midpoint potential of the Rieske protein strongly depends on the amino acids surrounding the iron–sulfur cluster as discussed in ***(5,7)***. By this theoretical analysis of a given amino acid sequenced, the range of the midpoint potential of the Rieske protein of interest can already roughly be estimated and its EPR characteristics after complete or partial reduction can be predicted.

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5

Heterologous Expression of Human Scavenger Receptor Class B Type I (SR-BI) in *Pichia pastoris*

Chang-Hoon Han, Moritz Werder, Wilk von Gustedt, and Helmut Hauser

1. Introduction

The advent of recombinant DNA technology made the overexpression of otherwise scarcely available proteins possible. The expression of recombinant proteins in *Escherichia coli* (*E. coli*) is characterized by large yields in a short period of time. The recombinant protein may account for 30–50% of the total *E. coli* protein. The expression of the extracellular domain (ECD) of human Scavenger receptor (SR-BI) in *E. coli* was reported to yield large quantities of denatured protein (16.7mg/g wet weight of *E. coli* cells) present in inclusion bodies ***(1)***. To the best of our knowledge the overexpression of native, glycosylated SR-BI has not been reported in the literature. Here, we describe the overexpression of human SR-BI and the ECD of this protein in the methylotroph *Pichia pastoris* (*P. pastoris*).

As a eucaryote this yeast cell has the advantage of higher eucaryotic expression systems such as proper protein folding, protein processing and posttranslational protein modification while yielding relatively large amounts of the protein of interest and still being as easy to manipulate as *E. coli*. The methods utilized for the expression of the wild-type protein and the truncated form of SR-BI, the extraction, purification, and the characterization of the recombinant proteins are discussed.

2. Materials

2.1. Plasmid Constructions

1. Expression vectors pPICZB and pPICZαC (Invitrogen; Paisley, U.K.).
2. mRNA isolation kit (Oligotex Direct mRNA mini kit; QIAGEN; Hilden, Germany).

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3. Oligonucleotide primers (Dept. Chemistry, ETH Zurich).
4. *Taq* DNA polymerase, restriction enzymes (*Kpn1*, *Cla*1, *Xba*1), and T4 DNA ligase (Amersham; Freiburg, Germany).
5. 5'/3' RACE (rapid amplification of cDNA ends) kit and protease inhibitor tablets (Roche Applied Science; Mannheim, Germany).

2.2. Preparation of Spheroplasts from P. pastoris

1. P. pastoris (Invitrogen).
2. Components of media: yeast extract, peptone, D-sorbitol, dextrose, sucrose, yeast nitrogen base, biotin, Tris-HCl, $CaCl_2$ (Sigma; Buchs, Switzerland).
3. Yeast extract peptone dextrose (YPD) medium containing 1% yeast extract, 2% peptone, and 2% dextrose.
4. YPD plates containing 1% yeast extract, 2% peptone, 2% dextrose, and 1% agar.
5. Regeneration dextrose (RD) medium: 1 *M* D-sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.005% amino acids.
6. RDB plates containing 1 *M* D-sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5} biotin, 0.005% amino acids and 1% agar.
7. Buffered glycerol-complex (BMGY) medium: 1% yeast extract, 2% peptone, 100 m*M* potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol.
8. Buffered methanol-complex (BMMY) medium: 1% yeast extract, 2% peptone, 100 m*M* potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.5% methanol.
9. Buffer A: 10 m*M* Tris-HCl pH 7.5, 0.7 *M* sorbitol, 30 m*M* 1,4-dithiothreitol (DTT; Sigma).
10. Lyticase (2 IU/μL) kindly provided by Dr. Howard Riezman of the Biocenter of the University of Basel;.
11. CaS buffer: 10 m*M* Tris-HCl pH 7.5, 10 m*M* $CaCl_2$, 0.7 *M* sorbitol.

2.3. Transformation of P. pastoris

1. 40% (w/v) polyethylene glycol 6000 (PEG 6000; Amersham; Freiburg, Germany).
2. CaT buffer: 20 m*M* Tris-HCl pH 7.5, 20 m*M* $CaCl_2$.
3. SOS medium: 0.7 *M* sorbitol, 0.3 × YPD medium, 10 m*M* $CaCl_2$.
4. 0.7 *M* sorbitol.
5. Zeocin™ (Invitrogen).

2.4. Expression of Recombinant Proteins in P. Pastoris

1. BMGY medium containing 100 μg/mL Zeocin.
2. Methanol.

2.5. Extraction and Purification of Recombinant Proteins

1. Nickel-Sepharose (QIAGEN; Hilden, Germany). The purchased slurry is diluted with 9 aliquots of wash buffer, followed by five washing steps using wash buffer.
2. Wash buffer: 50 m*M* Tris-HCl pH 7.8, 0.5 *M* NaCl.

3. Elution buffer: 50 m*M* Tris-HCl pH 7.8, 0.5 *M* NaCl, 0.5 *M* imidizole.
4. Breaking buffer: 50 m*M* Na^+-phosphate pH 7.4, 1 m*M* disodium salt of ethylenediaminetetraacetic acid (EDTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF; Sigma; Buchs, Switzerland), 5% glycerol, and 0.1% protease inhibitor tablet.
5. Acid-washed glass beads (Sigma; Buchs, Switzerland).
6. 25% (w/w) sucrose.
7. 35% (w/w) sucrose.
8. Phosphate-buffered saline (PBS), pH 7.4.

2.6. Characterization of the Recombinant Proteins

1. N-glycosidase F (New England Biolabs; Bioconcept, Allschwil, Switzerland), 500 NEB units/ μL.
2. Protein lysis buffer: PBS pH 7.4 containing 5% SDS and 10% β-mercaptoethanol (Sigma).
3. G7 buffer: 50 m*M* Na^+-phosphate pH 7.5.
4. 10% NP-40 (Sigma).
5. Sodium dodecyl sulfate (SDS) sample buffer: 0.25 *M* Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 160 m*M* (DTT) and 0.04 mg/mL bromophenol blue.
6. SDS polyacrylamide gel electrophoresis (SDS-PAGE) equipment: Mini-Protean II dual slab cell (Bio-Rad).
7. Egg phosphatidylcholine (Lipid Products; South Nutfield, U.K.).
8. Cholesteryl oleate (Sigma).
9. [1α, 2α(N)$^{-3}$H]cholesteryl oleyl ether, 58 Ci/mmol (Amersham; U.K.).
10. Anti-SR-BI antibody (pAb230) raised against the extracellular domain of mouse SR-BI (Novus Biological; AbCam, Cambridge, U.K.).
11. Anti-SR-BI antibody (pAb589) raised against the C-terminus of human SR-BI (Genosys; Cambridge, UK).
12. Anti-c-myc antibody (Santa Cruz Biotech; Santa Cruz, CA).

3. Methods

The methods described next outline: 1) the construction of the expression plasmids; 2) the preparation of spheroplasts from *P. pastoris*; 3) the transformation of *P. pastoris*; 4) the expression of the recombinant proteins; 5) the extraction and purification of recombinant proteins; and 6) the characterization of these proteins in terms of purity and enzymatic activity.

3.1. Plasmid Constructions

The construction of the expression plasmids for both the full-length SR-BI and the ECD of SR-BI are described in **Subheadings 3.1.1.** and **3.1.2.** This includes the description of the expression vectors, the cDNA inserts and the cloning.

3.1.1. Expression Vectors pPICZB and pPICZαC

Both expression vectors contain a methanol-inducible AOX1 (alcohol oxidase) (*see* **Fig. 1**) controlling the expression of alcohol oxidase ***(2)***, which catalyzes the first step in the methanol metabolic pathway. The *P. pastoris* expression system takes advantage of the powerful AOX1 promotor to drive high-level expression of recombinant proteins ***(3,4)***. In the absence of a carbon source such as glucose, *P. pastoris* can utilize methanol as a sole source of carbon and energy. The full-length sequence of human SR-BI (amino acids 1-509) and the truncated sequence L41-A421 comprising the ECD of human SR-BI are inserted into the multiple cloning sites of the expression vectors pPICZB and pPICZαC, respectively. Both expression vectors contain a Zeocin resistance gene for selection and a C-terminal polyhistidine $(His)_6$ tag for rapid purification on nickel-Sepharose and for possible detection of the recombinant proteins with an anti-*His* antibody. In addition, both vectors have a c-*myc* epitope for convenient detection of the recombinant proteins by Western blotting using an anti-c-*myc* antibody. Furthermore, the expression plasmid pPICZαC includes an N-terminal α-factor, a secretory signal, for efficient export of the protein into the cell medium (*see* **Fig. 1**).

3.1.2. Cloning

DNA manipulations are performed using standard recombinant DNA methods ***(5)***. Total mRNA is isolated from human enterocytes, and full-length cDNA of SR-BI is obtained by 5RACE ***(1)***. The cDNA of either the full-length SR-BI (amino acid residues 1-509) or the ECD of SR-BI (amino acid residues 41-421) is amplified by (polymerase chain reaction [PCR]; *see* **Note 1**). For PCR of full-length SR-BI (the ECD of SR-BI) the forward primer is designed to introduce a *Kpn*1(*Cla*1) site followed by the initiation codon and the reverse primer to introduce a stop codon and an *Xba*1 site (*see* **Fig. 1**). The PCR products are ligated into the corresponding cloning sites of pPICZB (for the expression of full-length SR-BI) and pPICZαC (for the expression of the ECD) (*see* **Fig. 1**). Prior to the transformation of *P. pastoris*, the expression constructs were verified by digestion with the appropriate restriction enzymes and DNA sequencing.

3.2. Preparation of Spheroplasts from P. Pastoris

The steps described here involve the preparation of spheroplasts from *P. pastoris*. Spheroplasts are more susceptible to transformation than the original cells surrounded by a protective cell wall, and spheroplasts are also used to measure lipid uptake, i.e., the catalytic competence of cells overexpressing full-length SR-BI. The method of spheroplast preparation used is an adaptation of Invitrogen's protocol ***(6)***.

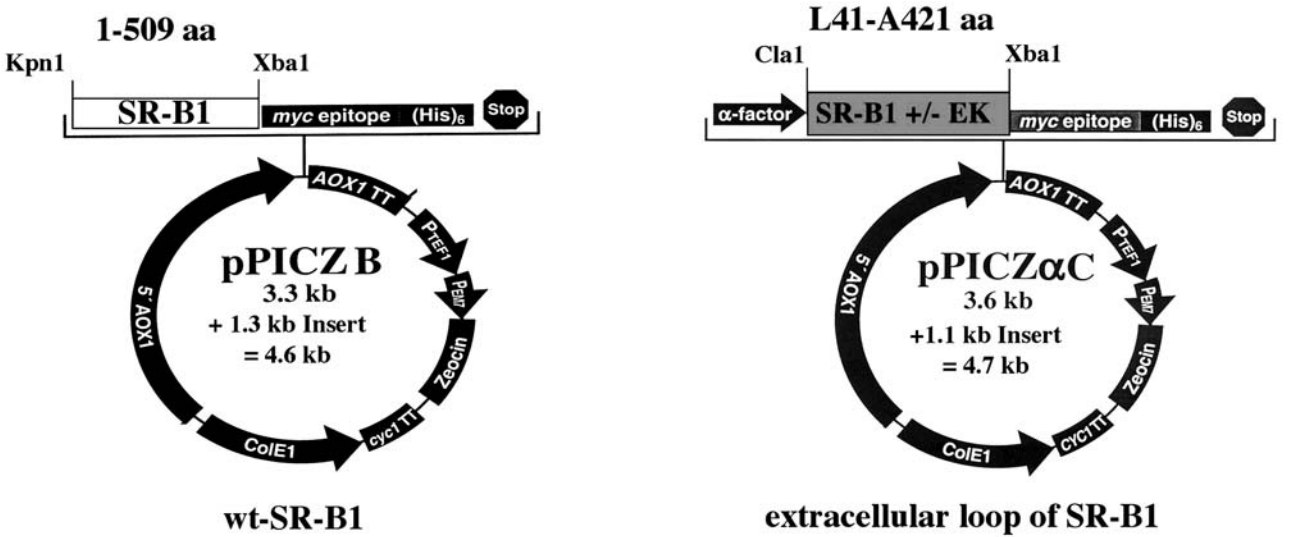

Fig. 1. Expression plasmids for the expression of recombinant SR-BI in *P. pastoris.* Schematics of the expression vectors pPICZB and pPICZαC for the expression of full-length SR-BI and the ECD of SR-BI, respectively. The expression vector pPICZαC is constructed either with or without an enterokinase (EK) cleavage site. The recombinant protein resulting from the vector containing the EK site can be digested with EK to remove the c-*myc* and the $(His)_6$ sequence. This sequence may affect the folding and activity of the protein expressed.

3.2.1. Cell Growth

1. Plate cells by streaking a glycerol stock of *P. pastoris* cells on a YPD plate and incubate at 30°C for 2 d. Isolated single colonies form.
2. Inoculate 10 mL YPD medium in a 50-mL conical flask with a single colony from the YPD plate and allow to grow overnight at 30°C under vigorous shaking (250 rpm). The cell culture may be stored at 4°C for several days.
3. Inoculate 200 mL each of YPD medium (in three 500-mL culture flasks) with 5, 10, and 20 µL of overgrown cells from the cell culture (*see* **step 2**) and incubate overnight at 30°C under vigorous shaking (250 rpm).
4. Monitor the optical density at 600 nm (OD_{600}) in each of the three cell cultures and harvest the cells from cultures with an $OD_{600} = 0.2 - 0.3$ by centrifugation at 1500*g* for 5–10 min at room temperature (*see* **Note 2**).

3.2.2. Cell Washing

1. Wash the cells by centrifugation at 1500*g* for 5 min at room temperature and resuspension in 20 mL sterile water by swirling the tube, and transfer the cell suspension to a sterile 50-mL conical tube.
2. Cells are washed again by centrifugation and resuspension by swirling in 20 mL of buffer A.
3. Repeat **step 2** and divide the cell suspension in buffer A into two portions of 10 mL each filled in two 50-mL conical tubes.

3.2.3. Lyticase Treatment (see ***Note 3***)

1. Fill 200 µL of cell suspension from the first sample tube (*see* **Subheading 3.2.2., step 3**) in a tube marked zero time point, add 5 µL lyticase (2 IU/µL) and determine immediately the optical density at 800 nm ($OD_{800,t=0}$).
2. Add 245 µL lyticase (490 IU) to the first sample tube, mix gently by inversion and incubate at 30°C. The suspension in the first sample tube is used to determine the optimal incubation time for spheroplast formation.
3. Withdraw 200 µL of cell suspension from the first sample tube at timed intervals of $t = 2, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45$, and 50 min of lyticase treatment and determine the optical density $OD_{800,t}$ for each time point t.
4. Calculate the percentage of spheroplast formation for each time point using the following equation ***(6)***: % spheroplasting $= 100 - [(OD_{800,t}/OD_{800,t=0}) \times 100]$.
5. In this way the average time $t_{70\%}$ of the lyticase treatment is ascertained that results in approx 70% spheroplast formation. Under the experimental conditions used (200 µL cell suspension $OD_{600} = 2–3$, in the presence of 10 IU of lyticase), the average incubation time $t_{70\%} = 23 \pm 7$ min (mean ± SD).
6. Spheroplasts are made from the cell suspension in the second 50 mL sample tube (*see* **Subheading 3.2.2., step 3**) by adding 5 µL of lyticase (10 IU) to 200 µL aliquots of cell suspension, mixing gently, and incubating at 30°C for about 20 min.
7. Harvest the spheroplasts thus formed by centrifugation at 750*g* for 10 min at room temperature.

8. Wash the pellet of spheroplasts once with buffer A by gently dispersing the pellet (by tapping the tube rather than vortexing) and pelleting the spheroplasts by centrifugation as described in **step 7** (*see* **Note 4**).
9. Wash the spheroplasts once with CaS buffer by centrifugation and gentle resuspension in 0.6 mL CaS buffer. For transformation spheroplasts must be used immediately, at least within 30 min. The yield of spheroplasts is sufficient for six transformations.

3.3. *Transformation of* P. Pastoris

The next step involves the transformation of spheroplasts with the expression plasmids.

1. Dispense 100 μL of spheroplast *suspension* (*see* **step 9,** above) into a sterile 15-mL centrifuge tube, add 10 μg plasmid DNA and incubate at room temperature for 10 min.
2. To induce transformation add 1 mL of a freshly prepared 1:1 mixture of a 40% PEG (polyethylene glycol) *dispersion* in H_2O and CaT buffer by gentle mixing.
3. Incubate the mixture at room temperature for 10 min and pellet the cells by centrifugation at 750*g* for 10 min at room temperature.
4. Remove the supernatant carefully by aspiration and invert the sample tube to drain residual supernatant.
5. Resuspend the pellet of transformed cells in 150 μL SOS medium and incubate at room temperature for 20 min.
6. Add 850 μL of 0.7 *M* sorbitol.
7. Plate a mixture of 200 μL of transformed cells and 10 mL of molten RD (regeneration dextrose) agarose onto (regeneration dextrose base RDB) plates containing Zeocin (100 μg/mL) and incubate the plates at 30°C. Zeocin-resistant transformants appear after 4–6 d.

3.4. *Expression of Recombinant Proteins in* P. Pastoris

The next steps describe the induction with methanol to initiate protein expression and the harvesting of the resulting recombinant proteins.

1. Inoculate 25 mL BMGY medium containing Zeocin (100 μg/mL) with a single colony of transformed cells in a 250-mL baffled flask and allow to grow to an optical density OD_{600} = 2–6 at 30°C in a shaking incubator (250 rpm).
2. After 16–18 h (cells are in the logarithmic growth phase) harvest the cells by centrifugation at 1500*g* for 5 min at room temperature.
3. Resuspend the cells to an OD_{600} = 1 in BMGY medium (about 100–200 mL), transfer the suspension to a 1-L baffled flask and cover with a sterile cotton plug.
4. Add 100% methanol to a final concentration of 0.5% at time 0, 24, and 48 h to maintain induction of protein expression.
5. At timed intervals (0, 6, 12, 24, 36, 18 h) transfer 1 mL of the expression culture to a 1.5-mL microcentrifuge tube, pellet the cells by centrifugation at maximum speed in a microcentrifuge for 2–3 min at room temperature and measure the level of

expression by SDS-PAGE and Western blotting using pAb230. In this way the optimal expression time, the time between induction of SR-BI expression and harvesting of cells, is determined.
6. Harvest cells overexpressing the full-length SR-BI by decanting the supernatant and freezing the cell pellet at −80°C using liquid N_2 or a dry ice/alcohol bath. These cells are used for the production of spheroplasts (*see* **Subheading 3.2.**) and the preparation of a total membrane fraction (*see* **Subheading 3.5.2.**).
7. For harvesting the ECD of SR-BI excreted into the culture medium, pellet the cells and collect the culture medium, i.e., the supernatant, in a separate vessel and adjust the pH to 8.0 by adding 0.5 *M* NaOH.

3.5. Extraction and Purification of Recombinant Proteins

As shown in **Subheading 3.6.**, the full-length SR-BI as an integral membrane protein is located in the plasma membrane of *P. pastoris*. In contrast, the ECD of SR-BI is secreted into the cell medium.

3.5.1. Isolation and Purification of the ECD of SR-BI

1. Add 2 mL Ni^{2+}-Sepharose slurry to 100 mL of the supernatant (*see* **Subheading 3.4., step 7**) and incubate the mixture on a turning wheel for 1 h at 4°C (*see* **Note 5**).
2. Wash the slurry of Ni^{2+}-Sepharose beads three times with several volumes of wash buffer.
3. Elute proteins bound to Ni^{2+}-Sepharose with elution buffer. Proteins thus eluted are analyzed by SDS-PAGE, Western blotting, and activity tests (*see* **Subheading 3.6.**). The purity of the protein varies, but greater than 91% pure protein can be obtained by this single-step purification.

3.5.2. Isolation and Enrichment of Full-Length SR-BI

For the isolation and enrichment of full-length SR-BI, a total membrane fraction is prepared from cells of *P. pastoris* according to Villalba et al. *(7)*. Cells overexpressing this protein are broken by vigorous shaking with glass beads and a total membrane fraction is prepared from these cells for the characterization of full-length SR-BI by SDS-PAGE and Western analysis.

1. Harvest cells overexpressing full-length SR-BI from 50 mL of culture by centrifugation at 3000*g* for 10 min at 4°C yielding a cell pellet of 2.5–3 g wet weight. Alternatively, an equivalent amount of frozen cells (*see* **Subheading 3.4., step 6**) is thawed and used.
2. Disperse the cells (2.5–3 g) in 2.5 mL breaking buffer followed by centrifugation at 3000*g* for 10 min at 4°C.
3. Resuspend the cell pellet in 2.5-mL breaking buffer and add 5 mL acid-washed glass beads (0.5 mm diameter), vortex the mixture for 30 s, and incubate on ice for 30 s.
4. Vortexing and incubation on ice are repeated at least eight times.

5. Centrifuge the mixture at 3000*g* for 10 min (in a Sorvall SS-34 rotor) to remove the glass beads and cell debris.
6. Centrifuge the supernatant resulting from **step 5** at 200,000*g* for 60 min (in a Beckman 50 Ti rotor) to obtain the "total membrane" fraction.
7. Apply the total membrane fraction from about 2.5 g of cells to a discontinuous sucrose gradient made of 1 mL 25% (w/w) sucrose and 1 mL of 35% (w/w) sucrose.
8. Centrifuge at 200,000*g* (in a Beckman TLS 55 rotor) overnight and collect the plasma membrane fraction enriched at the 25%/35% sucrose interface with a Pasteur pipet.
9. Dilute with 4 vol of water and pellet the plasma membrane fraction by centrifugation at 200,000*g* (in a Beckman 40 Ti rotor) for 20 min.
10. Resuspend the pellet in 0.6 mL PBS (pH 7.4) for deglycosylation, SDS-PAGE, and Western blotting of the proteins.

3.6. Characterization of the Recombinant Proteins

3.6.1. Deglycosylation of Proteins

Deglycosylation of the recombinant proteins is performed with N-glycosidase F (New England Biolabs) following the manufacturer's protocol. Briefly, 5 μL of protein lysis buffer are added to 45 μL protein dispersion in PBS buffer (2 mg protein/mL) and the sample is boiled at 100°C for 10 min. The denatured protein sample (40 μL) is mixed with 5 μL of G7 buffer, 5 μL of 10% NP40 and 1 μL N-glycosidase F ***(8,9)***. The mixture is incubated at 37°C for 1 h and an equal volume of SDS sample buffer is added. SDS-PAGE using silver staining ***(10)*** is performed according to the Bio-Rad instruction manual, Western blotting as described previously ***(11)***.

3.6.2. Catalytic Competency of the Recombinant Proteins

First and foremost, the question is addressed whether the recombinant proteins are properly folded and biologically active. In order to assess the biological activity of the ECD of SR-BI, the binding of apolipoprotein A-I (apo A-I), a well-known ligand of SR-BI, is measured using an apo A-I overlay technique described previously ***(11)***. For assessing the biological activity of full-length SR-BI, the uptake of cholesteryl ester (CE) by spheroplasts is determined using small unilamellar egg phosphatidylcholine vesicles as the donor. The CE uptake measurements are carried out as described in previous publications from our laboratory ***(1,11–13)***. The donor vesicles contain 0.15 mol% cholesteryl oleate radiolabeled with tritiated cholesteryl oleyl ether as an unhydrolyzable analogue of the cholesteryl ester (for the preparation of small unilamellar lipid vesicles (*see* **refs. *1,12,13***). Spheroplasts from *P. pastoris* overexpressing full-length SR-BI are used as lipid acceptor. As a control, spheroplasts are used that are made from either untransformed cells or transformed cells lacking methanol, i.e., uninduced cells not expressing SR-BI. At time zero 60 μL of

donor vesicles dispersed in buffer A (100 μg lipid/mL, 1.5 μCi/mL) are mixed with 600 μL of spheroplast suspension in buffer A (OD_{600} = 1), and the mixture is incubated at room temperature for 4 h. Donor and acceptor are then separated by centrifugation as described earlier and the radioactivity in both donor and acceptor is determined in a Beckman LS 7500 scintillation counter.

3.6.3. Characterization of the ECD of SR-BI

The results of the SDS-PAGE and Western analysis are summarized in **Fig. 2**. The SDS-PAGE pattern of the glycosylated ECD of SR-BI is an uninterpretable smear of bands. Upon deglycosylation of the protein the unresolved pattern is replaced by well resolved bands (*see* **Fig. 2C**, lanes 1 and 2) indicating that the lack of resolution is due to the glycosylation of the protein. The SDS-PAGE pattern of the deglycosylated ECD consists of a major band at 52 kDa and three minor bands (*see* **Fig. 2C**, lane 2): one band at about 120 kDa assigned to residual glycosylated protein, one at 38 kDa just below the 52 kDa band that can be assigned to N-glycosidase F, and a third one at 28 kDa. This band is either an impurity or a degradation product of the ECD of SR-BI. Densitometry of the SDS-PAGE pattern (*see* **Fig. 2C**, lane 2) indicates that the minimum purity of the ECD of SR-BI is 95%.

Western blots of the glycosylated ECD of SR-BI using two different antibodies (the anti-c-*myc* antibody and the anti-SR-BI antibody pAb230) show diffuse bands centered at about 120 kDa (*see* **Fig. 2A,B**). Upon deglycosylation using N-glycosidase F, the diffuse band at 120 kDa disappears or its intensity is greatly reduced, and a single, sharp band at 52 kDa appears (*see* **Fig. 2A,B**). It is evident from deglycosylation that the diffuse band centered at 120 kDa is owing to protein molecules differing in the degree of glycosylation. The apparant molecular weight of the deglycosylated protein derived from SDS-PAGE and Western analysis (*see* **Fig. 2**) is in excellent agreement with the value of 52.1 kDa calculated from the amino acid composition of the ECD of SR-BI. In contrast, the diffuse band at 120 kDa assigned to the glycosylated ECD is apparently caused by dimerization. This is evident from a simple calculation based on the molecular weights of deglycosylated full-length SR-BI (M_r = 57,000) and the deglycosylated ECD of SR-BI (M_r = 52 100) derived from the amino acid composition and on the apparent M_r values of the monomeric and dimeric forms of glycosylated, full-length SR-BI. These apparent M_r values were derived from SDS-PAGE ***(11)*** and the values used in the calculation are 84,000 and 145,000, respectively. Apparently, deglycosylation leads to the dissociation of the dimer.

Furthermore, apo A-I overlay experiments using the blotted membranes (*see* **Fig. 2A,B**, lane 1) yield a diffuse band centered at 120 kDa entirely consistent with Western blot analysis (data not shown). The fact that apo A-I, a natural lig-

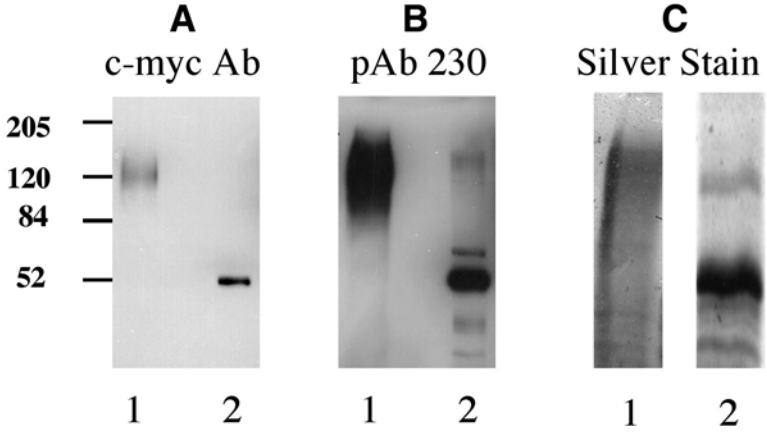

Fig. 2. SDS-PAGE and Western blot analysis of the ECD of SR-BI expressed in *P. pastoris*. The protein isolated and purified as described in **Subheading 3.5.1.** is resolved on 10% SDS-PAGE and transferred to nitrocellulose for Western blot staining with anti-c-*myc* antibody (c-myc Ab) (**A**) and anti-mouse SR-BI IgG (pAb230) (**B**). The original silver-stained SDS-PAGE pattern is shown in (**C**). The fully glycosylated form of the protein is applied in lanes 1, the deglycosylated protein in lanes 2. The position of molecular mass markers in kilodaltons is given on the left side of panel A.

and of SR-BI, is bound to the ECD of SR-BI is taken as an indication that this protein is properly folded. As a secretory protein, the ECD of SR-BI is secreted into the cell medium. This observation lends further support to the notion that the ECD is properly folded and biologically active. An unfolded or misfolded protein is bound to be either retained in the endoplasmic reticulum or to aggregate in the cytosol. This is clearly not the case.

3.6.4. Characterization of Full-Length SR-BI by SDS-PAGE and Western Blot Analysis

The results of SDS-PAGE and Western blotting of the plasma membrane fraction of *P. pastoris* overexpressing full-length SR-BI are shown in **Fig. 3**. Similar results are obtained by Western blotting using either the anti-c-*myc* antibody or the antihuman SR-BI antibody (pAb589). Both antibodies give four bands at about 84 kDa, 145 kDa, 209 kDa, and 237 kDa in order of increasing approximate molecular mass. The band at an apparent molecular mass of 84 kDa is characteristic of fully glycosylated SR-BI. This conclusion is supported by deglycosylation using N-glycosidase F: the 84 kDa band disappears or its intensity is greatly reduced and a new band at 57 kDa appears which is characteristic of the sugar-free form of SR-BI ***(1)***. The molecular mass of 57 kDa is entirely consistent with the value calculated from the amino acid composition of SR-BI.

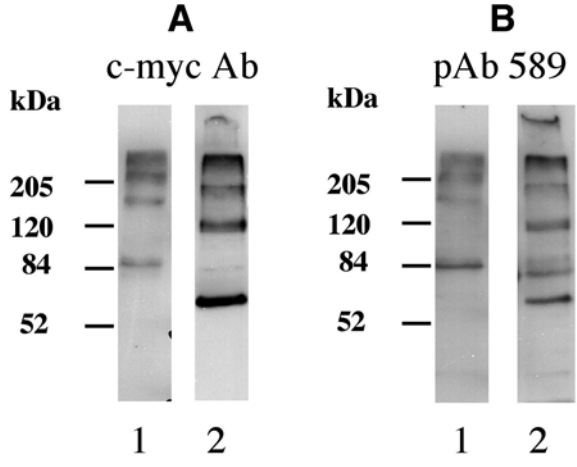

Fig. 3. Western blots of the total membrane fraction of *P. pastoris* cells overexpressing full-length SR-BI. The total membrane fraction is prepared as described in **Subheading 3.5.2.,** resolved on 10% SDS-PAGE and transferred to nitrocellulose for Western blot staining with c-*myc* Ab (**A**) and antihuman SR-BI IgG (pAb589) (**B**) as afore described ***(11)***. The fully glycosylated protein is applied in lanes 1, the deglycosylated protein in lanes 2. The position of molecular mass markers in kilodaltons is given on the left side of each panel.

Because upon deglycosylation all four bands of the Western blot (*see* **Fig. 3A,B**) move to lower molecular masses, these bands are assigned to glycoproteins. The four bands obtained by deglycosylation and detected with both antibodies are at 57 kDa, 120 kDa, 175 kDa, and 227 kDa (*see* **Fig. 3A,B** lanes 2). The high-molecular-weight proteins are apparently multiples of 57 kDa, the monomeric form of SR-BI. We conclude that human SR-BI expressed in *P. pastoris* has a tendency to aggregate within the plane of the plasma membrane (*see* **Fig. 3A,B**). A similar aggregation of SR-BI to dimers, trimers, tetramers, and higher oligomers can be detected by Western blotting of rabbit BBMV using pAb230 or pAb589 (data not shown). **Fig. 3** provides good evidence for the tendency of the membrane protein SR-BI to aggregate within the plasma membrane consistent with a previous report ***(11)***. It is, however, still unknown whether the monomeric form and/or the oligomeric forms are biologically active.

3.6.5. Biological Activity of Full-Length SR-BI Expressed in P. Pastoris

As a test of the biological activity of full-length SR-BI, the uptake of cholesteryl oleate (CE) is determined at room temperature using small, unilamellar egg phosphatidylcholine vesicles as the donor and spheroplasts as the acceptor. In order to make the plasma membrane of *P. pastoris* accessible to lipid donor parti-

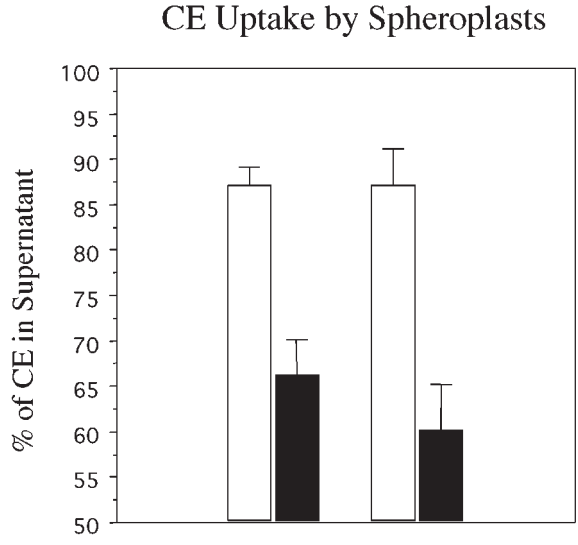

Fig. 4. Cholesteryl oleate (CE) uptake by two kinds of spheroplasts. CE uptake is measured as detailed under **Subheading 3.6.** Sheroplasts made from *P. pastoris* overexpressing full-length SR-BI are used as the CE acceptor (black bars). The CE uptake of these spheroplasts is compared to that of spheroplasts prepared from transformed, but uninduced cells, i.e., cells to which no methanol is added and hence no expression of SR-BI takes place (open bars).

cles, at least the partial removal of the cell wall is required. Intact cells of *P. pastoris* show no CE uptake. Spheroplasts are made from transformed cells in which overexpression of full-length SR-BI is induced by the addition of methanol as described in **Subheading 3.4.** As a control spheroplasts are prepared from either untransformed cells or transformed cells lacking methanol (uninduced cells). In spheroplasts from cells overexpressing SR-BI, the CE uptake exceeds that of control spheroplasts by a factor of 2.9 ± 0.2 (mean ± SD) (*see* **Fig. 4**). Both types of control cells behave similarly. We can conclude that spheroplasts made from cells overexpressing SR-BI exhibit selective CE uptake closely resembling the CE uptake observed with brush border membrane vesicles ***(11)***. The results in **Fig. 4** are good evidence for SR-BI (expressed in the plasma membrane of *P. pastoris*) being biologically active and, hence, properly folded. The determination of the optimal expression time, the time between induction of SR-BI expression and harvesting of cells, is important. Starting with a cell suspension of OD_{600} = 1 an expression time of less than 4 h is insufficient to detect SR-BI-mediated CE uptake. The optimal expression time is about 6 h. Prolonged expression has the effect of reducing CE uptake relative to optimal expression. For instance, spheroplasts pro-

duced from cells cultivated for 24 h exhibit no SR-BI-specific CE uptake. Freeze–fracture electron microscopy (EM) indicates (data not shown) that spheroplast formation from these cells is negligible. Apparently, the thickness of the cell wall increases with expression time, and at times exceeding 6 h the digestion of the cell wall by lyticase becomes increasingly ineffective.

4. Notes

1. For PCR, the primer is designed to have a Kozak consensus sequence for proper translational initiation, for example ACC *ATG* G where ATG corresponds to the initiation codon of the gene of interest ***(14–16)***.
2. If the OD_{600} of the cell culture is over 0.3 (*see* **Subheading 3.2.1., step 4**) dilute the culture 1:4 with fresh medium and incubate at 30°C for 2–4 h until the OD_{600} is between 0.2 and 0.3.
3. For optimizing the lyticase treatment (*see* **Subheading 3.2.3.**), it is important to determine the minimum time required to produce the percentage of spheroplasts needed. Prolonged incubation with lyticase can be deleterious resulting in low transformation efficiency and eventually in cell lysis.
4. Lyticase digests the cell wall of *P. pastoris* and makes the cells extremely fragile. Gentle handling of the cell suspension is imperative.
5. For the purification of the ECD of SR-BI secreted into the medium, it is important to adjust the medium to pH 8.0 before adding nickel-Sepharose.

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6

Expression of Oligomeric Amiloride-Sensitive Epithelial Sodium Channel in Sf9 Insect Cells

U. Subrahmanyeswara Rao

1. Introduction

Elucidation of the structure and function of proteins isolated from cell lines or tissue source is limited by their lower abundance. Thus, it is a common practice to utilize a heterologous expression systems such as *Escherichia coli* (*E. coli*), yeast, mammalian, and Sf9 cells for the large-scale production of the protein so as to carry out detailed structure-function relations on the expressed protein ***(1–4)***. In most cases, the expressed recombinant proteins are monomeric and function independently without association with other proteins. However, the scenario is different for multimeric proteins, which require coexpression of all the subunits to elicit their functional characteristics ***(5–8)***. Similarly, although certain proteins, involved in cell signaling, for instance, may independently be expressed, the biological effects of the expressed proteins can only be ascertained by their interactions with other proteins. Production of biologically active multimeric recombinant proteins has been difficult because of a lack of simple procedures for the coexpression of all the required subunits in each of the expressing host cell. Whereas expression of such multimeric proteins in yeast can be accomplished easily as a result of the availability of multiple auxotrophic yeast strains and vector DNAs, the formidable and inefficient yeast cell-wall breakage procedures greatly reduce the recovery of the expressed proteins to unacceptable levels for any structural or functional analyses ***(9)***. The focus of our laboratory is to understand the amiloride-sensitive epithelial Na^+ channel (ENaC)-mediated Na^+ transport ***(10,11)***. ENaC is a multimeric protein composed of three subunits termed, α-ENaC, β-ENaC, and γ-ENaC ***(12)***. Although yeast is well suited for the expression of ENaC complex, ENaC subunits could not be detected in stably transformed yeast clones in our laboratory.

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Thus ENaC has provided us with an opportunity to explore ways to express the multimeric membrane protein in Sf9 insect cells in amounts sufficient for structural studies.

The Sf9 insect cell-baculovirus expression system is considered as an efficient low-cost protein factory ***(13)***. This expression system uses baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), to express foreign proteins in cells derived from the ovary of the fall armyworm, *Spodoptera frugiperda* (Sf9 insect cells) ***(14)***. The AcNPV genome contains several genes (e.g., polyhedrin and *P10*) that are nonessential in the tissue culture life cycle of the baculovirus and can be replaced with the genes of interest. However, the AcNPV genome is >130 kb and is not amenable for direct subcloning of the cDNA of interest. Thus, the genes to be expressed are subcloned first into specialized vector DNA (e.g., pVL1393 transfer vector), which are specific for the construction of recombinant baculoviruses. Two important features of these vectors are the presence of recombination sequences flanking the multiple cloning sites and strong baculoviral promoters, which facilitate the transfer of cDNA along with the promoter into the baculoviral genome and high-level expression, respectively. Cotransfection of the transfer vector carrying the cDNA and wild-type baculoviral DNA into the host Sf9 insect cells results in the recombination between homologous sites in these two DNAs, transferring the cDNA along with the promoter from the vector to the baculoviral genome. Owing to the use of slightly modified baculoviral genome (e.g., linearized) in these experiments, only the recombinant baculoviruses are packaged in the host cell, which are released into the culture medium. The culture medium thus contains highly homogenous baculoviral particles, which is directly used as recombinant baculoviral stock for further infection of Sf9 insect cells. Infection of Sf9 insected cells with this recombinant baculoviral stock usually results in the high yield expression of foreign protein. It must be noted that Sf9 cells carry out most of the posttranslational modifications, which normally occur in the mammalian cells.

A survey of the literature has pointed out that the expression of multimeric proteins in Sf9 insect cells is accomplished by coinfection with multiple baculoviruses, each harboring the required subunit cDNA. To coexpress all of the three ENaC subunits, we have first followed this popular strategy of infection of Sf9 cells with three recombinant baculoviruses carrying the individual subunit cDNAs. Our data indicate that the amount of ENaC expressed in this manner is very low. In addition, most of the Sf9 insect cells infected with this pool of three baculoviruses appeared to be expressing only one subunit, suggesting that an Sf9 cell infected with one baculovirus may become immune to further infection with other baculoviruses. Thus, this experimental procedure appeared inefficient in the production of ENaC as a complex. To fully utilize the poten-

tial of the Sf9 insect cell-BV expression system, we have examined several strategies to coexpress all of the three ENaC subunits in Sf9 cells. This resulted in the development of a procedure in which the most commonly used pVL1393 transfer vector was manipulated to carry all of the three subunit cDNAs, each of which is under the control of a polyhedrin promoter. A recombinant baculovirus generated using this transfer vector was able to induce high-level production of all ENaC subunits in each infected Sf9 insect cell. This strategy of preparing a transfer vector carrying multiple cDNAs is found to be applicable to the coexpression of multimeric proteins in mammalian cells as well (unpublished data).

2. Materials

1. Baculoviral Transfer Vectors (*see* **Note 1**). The first step in the expression of foreign proteins in Sf9 insect cells involves the subcloning of cDNA into a baculoviral transfer vector. A number of transfer vectors are available commercially, which differ mainly in their size, number of promoters, and the multiple cloning sites. The most commonly used pVL1393 transfer vector that contains a single strong polyhedrin promoter is useful. The quality of the vector DNA preparation is critical for the success of the experiment. The vector DNA should be prepared either by the cesium chloride/ethidium bromide density gradient centrifugation or by the commercially available plasmid DNA preparation kit procedures.
2. ENaC cDNAs. The plasmids carrying the ENaC cDNAs should also be of high quality, and should be prepared using the aforementioned procedures (*see* **Subheading 2.1.**). If the ENaC cDNAs are in propagation vectors such as pBluescript, these cDNAs should be subcloned into pVL1393 vector, under the control of polyhedrin promoter. The ENaC subunit cDNAs in our laboratory were first subcloned in the multiple cloning sites of pVL1393 transfer vector and generated pVL1393/αENaC, pVL1393/βENaC, and pVL1393/γENaC all of which are under the expression control of polyhedrin promoter.
3. The Sf9 insect cells and growth medium (*see* **Note 2**). Although several insect cell lines susceptible to baculoviral infection are available, the Sf9 cell line is commonly used. The Sf9 insect cells do not require CO_2 for growth and can grow at room temperature. These cells can be maintained as suspension culture in spinner flasks containing Grace's medium supplemented with 10% fetal bovine serum (FBS) at a density of 1×10^6/ml, which can be accomplished by splitting the cultures three times/wk with fresh growth medium. The cells can be made adapted to serum-free medium, which is commercially available. Because of the cost, we do not see any advantage of the use of this medium in the ENaC expression. The doubling time of these cells is approx 18 h at 27°C.
4. Baculoviral DNA. The commercially available linearized AcNPV DNA, is superior to the wild-type DNA isolated from AcNPV, in that the recombinant baculoviruses prepared from using the linearized AcNPV DNA do not require plaque purification.
5. Buffer A. Grace's medium supplemented with 10% FBS.

6. Buffer B. 25 m*M* HEPES, 125 m*M* $CaCl_2$, 140 m*M* NaCl, pH 7.1, sterilized by filtration.
7. Tissue culture supplies. 60-mm tissue culture plates; sterile 1.5-mL microcentrifuge tubes; 12-well tissue culture plates.

3. Methods

Construction of recombinant baculovirus involves two steps:

1. Preparation of baculoviral transfer vector carrying all three ENaC cDNAs and
2. Cotransfection of Sf9 insect cells with transfer vector and baculoviral DNA.

3.1. Construction of Bicistronic Transfer Vector, pVL1393/αβ

1. Subclone the α, β and γ cDNAs into the multiple cloning site region in the pVL1393 transfer vector and generate pVL1393/α, pVL1393/β, and pVL1393/γ transfer vectors (*see* **Fig. 1**).
2. Make certain that the orientation of the inserted cDNA is correct so that the expression of cDNA will be under the control of polyhedrin promoter. Note the presence of a restriction site *NaeI* in the recombination sequence, upstream to the polyhedrin promoter, which should be a unique site and available for digestion of the vector.
3. Linearize the pVL1393/β by digesting with any restriction site available at the 3'-end of the β cDNA. In our experiments, we have linearized with *Bgl*II.
4. If necessary, fill in the 3'-end with Klenow enzyme to make both ends blunt.
5. Purify this linearlized vector by using the commercially available kits and save.
6. Digest the pVL1393/α with *NaeI* (in the recombination sequence upstream to the polyhedrin promoter) and a site at the 3'-end of the α cDNA. If required, blunt this latter site with Klenow.
7. Isolate the released approx 3-kb fragment with both ends blunted, containing the polyhedrin promoter and the entire α cDNA, termed PHDRN-α by using the commercial kits.
8. Ligate the PHDRN-α with the linearized pVL1393/β that was also blunted, with T4 DNA ligase enzyme.
9. Follow the standard molecular biological procedures of transformation into *E.coli,* and isolation of plasmid DNA from the clones.
10. Carry out the restriction analyses on the plasmid DNAs prepared. Because this is blunt-end ligation, it is possible that the PHDRN-α could be inserted in pVL1393/β either in the right or reverse orientation. Use only the plasmid in which the PHDRN-α is oriented in right orientation, as shown in **Fig. 1**. Thus, the β and α cDNAs are present tandemly in the vector, which is called pVL1393/βα.

3.2. Construction of Tricistronic Vector, pVL1393/αβγ

1. Digest the pVL1393/γ with *NaeI* and a site at the 3'-end of the γ cDNA, and blunt this site if required, as described in **Subheading 3.1.**
2. Isolate the approx 3-kb fragment containing the polyhedrin promoter and the entire γ-cDNA, termed PHDRN-γ.

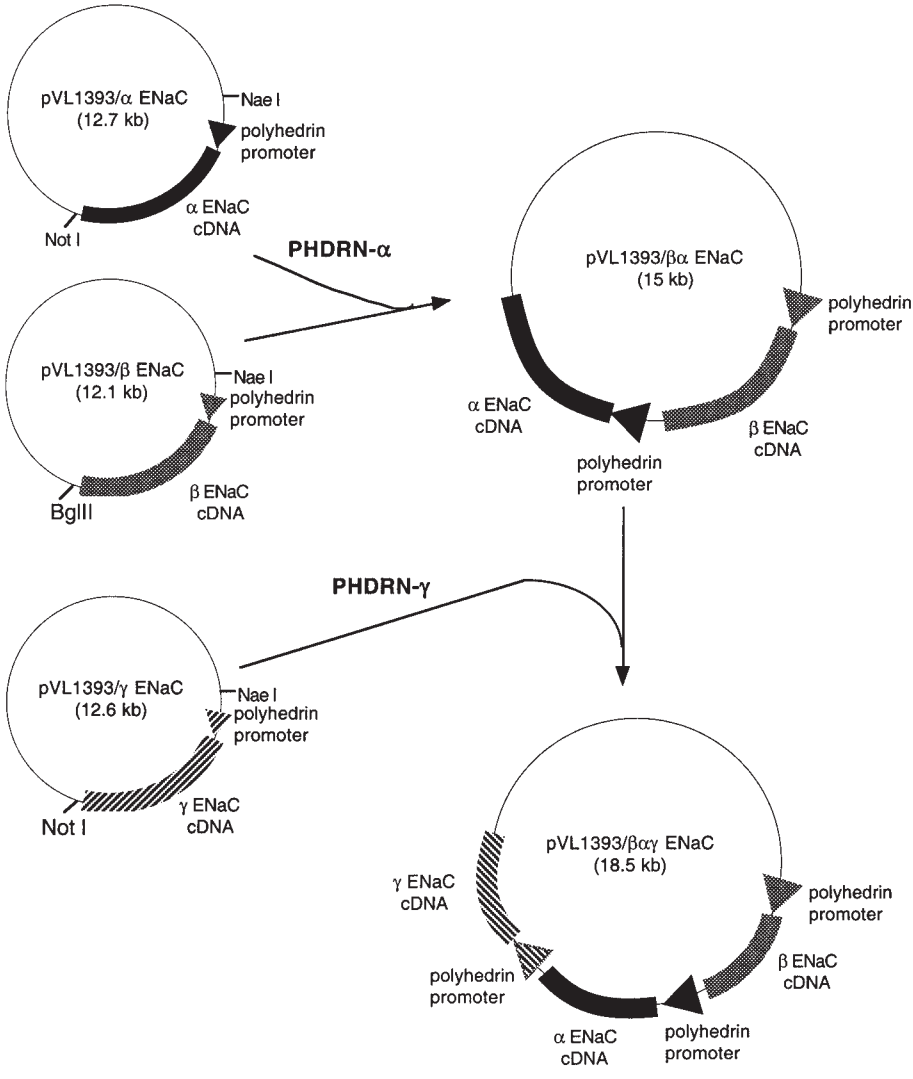

Fig. 1. Schematic representation of the construction of pVL1393 vector carrying three ENaC subunit cDNAs. The pVL1393/αENaC, pVL1393/βENaC, and pVL1393/γENaC are first constructed. The αENaC along with the polyhedrin promoter (PHDRN-α) was then transferred to pVL1393/βENaC vector, to generated pVL1393/βα. The γENaC cDNA along with the polyhedrin promoter (PHDRN-γ) was transferred to pVL1393/βα to generate pVL1393/βαγ.

3. Digest the pVL1393/βα prepared in **Subheading 3.1.** with a unique restriction enzyme available at the end of the α cDNA, and blunt it if necessary.
4. Ligate the PHDRN-γ with the linearized pVL1393/βα DNA, and follow the standard procedures of transformation and isolation of clonal DNA.
5. Select the clone in which the PHDRN-γ is inserted in the right orientation. Designate this plasmid as pVL1393/βαγ. In this vector, all three ENaC cDNAs are in tandem and each is under the transcriptional control of a polyhedrin promoter.

3.3. Cotransfection of Sf9 Insect Cells with Plasmid DNA

In this step, the linearized baculoviral DNA is mixed with a baculoviral transfer vector carrying the ENaC cDNAs (*see* **Subheading 3.2.**) in the presence of calcium. The DNA–calcium mixture is added to Sf9 insect cells attached to the tissue culture plates.

1. Use two 60-mm culture plates, one for the cotransfection and the other for negative control. Seed 2 million exponentially growing Sf9 insect cells from spinner flask in each culture plate, at least 1 h before the transfection experiment (*see* **Subheading 3.5**). The cells should be attached to the culture place within 5 min. The dead cells do not stick to the plate. If the percent of dead cells is more, discard the plate. Use only the cultures that contains nearly 95% healthy cells.
2. In a sterile 1.5-ml microcentrifuge tube, combine 0.5 μg of linearized baculoviral DNA and 5 μg of transfer vector (*see* **Subheading 3.2.**). As a negative control, avoid the addition of either one of these two DNAs in the mixture. Total volume should be less than 75 μL. Mix gently and leave the mixture at room temperature for 15 min.
3. Add 1 mL of Buffer B to each 1.5-mL microcentrifuge tube containing DNAs and leave them at room temperature for an additional 5 min.
4. Aspirate the medium in the 60-mm culture plates in which the Sf9 insect cells are firmly attached. Wash the cells twice gently with Buffer A (section 2.5). Finally add 1 ml of Buffer A to each plate.
5. Using a 200-μL pipetman, add the DNA-calcium mixture (*see* **step 3**), dropwise to the corresponding plate prepared in **step 4.** The distance between the pipet tip to the surface of the plate should be about 10 cm. Gently swirl the plate after each drop of DNA plummeted, to ensure proper mixing and formation of DNA-calcium phosphate precipitate. At the end of DNA-calcium mixture addition, the culture medium will turn slightly whitish, indicating the formation of calcium phosphate precipitate.
6. Transfer the plates into a Rubbermaid box, cover the lid, and incubate at 27°C for 4 h.
7. Aspirate the medium from the plates. Gently wash the cells twice with Buffer A (3 mL each wash). Add 3 mL of Buffer A to each plate.
8. Transfer the plates back to the Rubbermaid box, in which a few wet Kimwipe wipers are placed and incubate at 27°C for 4 d. The purpose of wet wipers is to prevent drying of the Grace's medium in the plates. Check every day and, if required, wet the wipers with distilled water.
9. After incubation for 4 d, aseptically transfer the medium from each plate to sterile 15-mL centrifuge tubes. Centrifuge at 3000*g* for 5 min. Carefully collect the super-

natants and save at 4°C. This is the First transfection fluid, and contains the recombinant baculovirus particles released form Sf9 insect cells.

3.4. Analysis of ENaC Production and Preparation of Viral Stocks

1. Seed 30,000 fresh Sf9 insect cells in each well of a 12-well plate, with a final volume of 0.5 mL Grace's medium containing 10% FBS. Five wells are required for the analysis of each first transfection fluid. Add 0, 1, 10, 50, and 100 μL from each first transfection fluid to each well, and incubate for 3 d in a 27°C incubator.
2. Collect the supernatants from each well and save, which are the first amplification supernatants. Add 50 μL of Laemmli disaggregating buffer to each well to dissolve the infected Sf9 insect cells. Collect the cell lysates in 1.5-mL microcentrifuge tubes and centrifuge at 14,000*g* for 10 min. Do not heat these samples, as heating promotes aggregation of membrane proteins.
3. Set up the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Load 10 μL from each cell lysates in the wells of SDS-acrylamide gel, and electrophorese the proteins. Electrotransfer the proteins from the gel onto a PVDF membrane, and probe the blots with appropriate antibodies, by following the standard procedures of Western blotting.
4. Determine the wells of 12-well plate in which ENaC subunits are overexpressed by comparing the Western blots. Use 0.2 mL of these first amplification supernatants for further amplification of the virus by infecting 8×10^6 cells growing in T-75 tissue culture flask for 3 d. The supernatants obtained at this stage will have approx 2×10^8 virus particles/ml, and serve as baculoviral stocks.

4. Notes

1. The pVL1393 and other baculoviral transfer vectors, linearized baculoviral DNA and the Grace's medium are available at different commercial sources (PharMingen, Invitrogen, Life Technologies, Novagen, and others).
2. The Sf9 insect cell-baculovirus expression system offers several advantages in addition to the overproduction of foreign proteins. The Sf9 cells do not require any CO_2 incubator and grow rapidly at room temperature, eliminating the need for an expensive incubator. The Sf9 insect cells can also grow in serum-free medium, which is specifically advantageous in the production of secretory proteins. We supplement the growth media with antibacterial and antifungal agents, which do not appear to affect the baculoviral production or the protein expression levels. Because the cells can be maintained as suspension cultures in spinner flasks, we have compared the production of ENaC in suspension cultures with that of infections carried in tissue culture flasks. The results suggested that Sf9 insect cells in suspension cultures produce extremely low levels of recombinant proteins when compared to the cells attached to the tissue culture plate.

Acknowledgment

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Functional Expression of *His*-Tagged Rhodopsin in Sf9 Insect Cells

Giel J.C.G.M. Bosman, Jenny van Oostrum, Githa Breikers, Petra H.M. Bovee-Geurts, Corne H.W. Klaassen, and Willem J. DeGrip

1. Introduction

The structure/function relationship of G protein-coupled receptors constitutes one of the most challenging areas of the present postgenomic era. It is estimated that at least a quarter of all human proteins are membrane proteins, with most of them belonging to the superfamily of GPCRs. Not only the relative number of GPCRs, but especially the key roles they play in cell function—and thus their pharmacological importance—warrant the attention they are presently receiving as a major focus of research. Indeed, more than 50% of the presently marketed medication targets this GPCR superfamily. This confronts us with the fact that their activation mechanism is still poorly understood, owing to a serious lack of information on structure and structure/function relationships. For almost all GPCRs, only models are available for the prediction of affinity, specificity, and/or effect of putative (ant)agonists. Detailed information on structure and function is not only hampered by the fact that, being integral membrane proteins, GPCRs are notoriously difficult to purify, but also by the lack of natural sources in which GPCRs are abundantly present. The rod visual pigment rhodopsin, a prototype of a major subclass of the GPCRs, is the only GPCR that can be isolated from native tissue in sufficient amounts to allow biochemical and biophysical studies. However, for detailed studies on the complex structure/function relation at the molecular level, there is a need for functional expression of recombinant protein (*see* **Note 1**). In our experience, the baculovirus expression system is an excellent option for this purpose. This system combines ease to handle with the ability to perform the posttranslational modifications required for correct folding—and thus correct

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function—of membrane proteins (*see* **Note 2**). Optimized procedures for large scale expression and purification of visual pigments allow yields of up to 4 mg/L. Here we describe the experimental details for the production, purification and functional analysis of recombinant rhodopsin. These procedures are used as a starting point for the production of other visual pigments, and GPCRs in general.

2. Materials

2.1. Production of Recombinant Baculovirus

All components necessary for the generation of recombinant baculovirus can be obtained from commercial sources either separately, and/or as part of ready-to-go expression systems ***(1***; *see* **Note 3)**.

2.2. Cell Culture

Insect cells are cultured at a temperature of 27 ± 1°C and do not require CO_2 supplementation. TNM-FH medium consists of Grace's basal insect cell medium ***(1)*** supplemented with yeastolate (3.3 g/L), lactalbumin hydrolysate (3.3 g/L), and bovine serum albumin (fraction V; 5.5 g/L). This medium can be prepared from the individual components, but can also be obtained from various commercial sources in liquid and in powder form. For serum- and protein-free medium we routinely use InsectXpress medium (BioWhittaker); similar media can be obtained from other commercial sources.

2.3. Production and Analysis of Recombinant Rhodopsin

1. Buffer A: 6 m*M* PIPES, 10 m*M* ethylene diamine tetraacetic acid (EDTA), 2 μg/mL leupeptin, 2 m*M* dithioerythritol, pH 6.5.
2. Buffer B: 20 m*M* PIPES, 130 m*M* NaCl, 10 m*M* KCl, 3 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 0.1 m*M* EDTA (*see* **Note 4**), 1 μg/mL leupeptin, pH 6.5.
3. Buffer C: 20 m*M* *bis*-trispropane, 0.5 *M* NaCl, 20% glycerol (v/v), 5 m*M* β-mercaptoethanol, 1 m*M* histidine, 2 μg/mL leupeptin, 20 m*M* n-dodecyl-β-1-D-maltoside (DoM; Anatrace), pH 7.0.
4. Buffer D: 20 m*M* *bis*-trispropane, 15% glycerol (v/v), 0.5 *M* NaCl, 20 m*M* n-nonyl-β-1-D-glucoside (nonyl; Anatrace), 5 m*M* β-mercaptoethanol, 2 μg/mL leupeptin.
5. Buffer E: 20 m*M* *bis*-trispropane, 150 m*M* NaCl, 15% glycerol (v/v), 5 m*M* β-mercaptoethanol, 20 m*M* nonyl, 50 m*M* histidine.
6. Buffer F: 20 m*M* HEPES, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 0.2 m*M* DoM, 1 m*M* DTE, pH 7.4.

3. Methods

New batches of medium (TNM-FH, serum-free medium) and bovine serum albumin, as well as new batches of cells are routinely tested for their perfor-

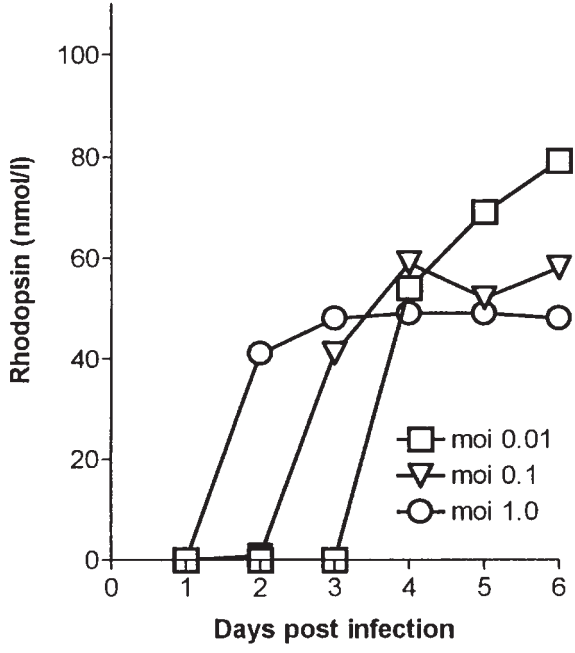

Fig. 1. Production of *His*6-tagged rhodopsin by Sf9 insect cells in spinner bottles. Spinner cultures were infected in their midexponential phase of growth at various MOIs. Rhodopsin yields were monitored for up to 6 h after infection. Note the significant larger production of functional pigment per unit volume for the lowest MOI (0.01). This appears to be largely caused by continuing cell division during the early phase of infection, whereas the net production per cell does not differ significantly for the three conditions.

mance with respect to cell growth and production of functional recombinant protein, before upscaling. For each recombinant protein we also determine the optimal multiplicity of infection (MOI) and optimal time of harvesting after infection (DPI) as required for maximal production of functional protein (*see* **Note 5**).

3.1. Production of Recombinant Baculovirus

We have used various procedures to generate recombinant baculovirus (***2–4***; *see* **Note 6**). The commercially available expression system we now routinely use (Bac-to-Bac® system; Life Technologies) is based on site-specific transposition of an expression cassette into a shuttle vector containing a somewhat adapted *Autographa californica* baculovirus genome (bacmid) that is propa-

gated in *Escherichia coli*. The recombinant bacmid DNA is used to transfect insect cells, resulting in the generation of a transfection supernatant containing recombinant baculovirus. These systems come with detailed protocols.

3.1.1. Preparation of a Virus Stock

Starting with a transfection supernatant (*see* **Subheading 3.1.**), we use at least one round of plaque purification prior to the production of a working virus stock:

1. In a 6-well culture dish, seed 8.10^5 Sf9 cells from a log-phase culture in 2 mL of TNM-FH medium per well (*see* **Subheading 2.2.**). Swirl the dish to distribute the cells evenly, and allow at least 15 min at 27°C to attach.
2. Remove the medium and replace with 0.6 mL TNM-FH.
3. Prepare tenfold dilutions of the transfection supernatant in TNM-FH (10^4–10^7), add 100 μL per well, and incubate for 1 h at 27°C.
4. Meanwhile, for one 6-well culture dish, autoclave 0.225 gm low temperature gelling agarose (Sea-Plaque agarose, FMC Bioproducts) in 7.5 mL of distilled water in a 50-mL polypropylene tube. Keep at 37°C in a water bath.
5. Add 6 mL 2× concentrated TNM-FH to the agarose, as well as 1.5 mL fetal calf serum and 7.5 μL of a concentrated antibiotics solution (streptomycin 50 mg/mL, penicillin 50.000 U/mL).
6. Remove the medium from the wells, and add 2 mL of the agarose overlay gently along the side of the well. Keep at room temperature for 30 min, and incubate for at least 5 h at 27°C in a humidified atmosphere.
7. Check for the presence of plaques after 5–10 d. Under a bright field microscope with the lamp switched off, plaques are visible as white cells on a background of darker, uninfected cells. If no plaques can be detected after 10 d, repeat the procedure with lower dilutions of the transfection supernatant.
8. For determination of the titer, MTT can be added to the well (1 mg/mL PBS); after 15–30 min, living cells form a colored product, whereas the plaques remain without color.
9. Place the microscope in a sterile hood, and pick 3–6 plaques into 0.5 mL TNM-FH using a pipet tip. Select the most isolated plaques from the highest positive dilution. Keep overnight at 4°C to elute the viruses from the agarose.
10. In a 6-well culture dish, seed 1.10^6 Sf9 cells in 2.0 mL TNM-FH per well. Add 200 μL of the eluted viruses and incubate at 27°C for 6 d.
11. Dislodge the cells from the well by pipet. Transfer to a sterile tube and centrifuge for 5 min at 3000*g*. Store the supernatant (P1) at 4°C. Use the cells for the detection of production of recombinant protein using appropriate methods (ELISA, dot blot, immunoblot).
12. Prepare a virus stock solution by infecting Sf9 cells, growing in midlog phase in 182 cm^2 culture flasks, using various amounts (1, 10, 100 μL) of P1. After 5–10 d, prepare the P2 working solution by centrifugation as aforementioned.

13. Determine the titer as described in **steps 1–8**. This will usually be around 1.10^8 PFU/mL.
14. Viral stocks can be stored at 4°C for at least 1 yr. A frozen stock (−80°C) can be used as a long-term backup ***(1)***.

3.2. Cell Culture

We routinely use the *Spodoptera frugiperda*-derived cell line Sf9 (ATCC CRL 1711) to amplify recombinant baculovirus and to express recombinant protein (*see* **Note 7**). The Sf9 cells are routinely cultured as a monolayer in TNM-FH medium (*see* **Subheading 2.1.**) supplemented with 10% heat-inactivated fetal calf serum (*see* **Note 8**) at a temperature of 27°C.

3.2.1. Maintenance of Insect Cells in Serum-Supplemented Medium

1. When the cells are 90% confluent, dislodge the cells from the flask by gentle pipet.
2. Dilute the cells 1:8 to 1:10 into a new flask. The cells will be ready to subculture again in 4 d. The volume of medium to be used is 5 mL, 10 mL, and 20 mL for T25, T75, and T182 flasks, respectively. The cell numbers that are reached at confluency are 5.10^6–10.10^6 cells for T25 flasks, 20.10^6–30.10^6 cells for T75 flasks, and 40.10^6–60.10^6 cells for T182 flasks.
3. Cultures of 100 mL are maintained in spinner bottles (Bellco) with an adjustable hanging bar, and are used for production of microgram amounts of protein. To inoculate a spinner bottle, cell densities as low as 1.10^5 cells/mL can be used when cells are growing (and kept) in serum-supplemented medium. For production of milligram amounts of protein, large-scale cultures are grown in stirred bioreactors (*see* **Subheading 3.2.3.**). In spinner bottles and bioreactors Pluronic® F-68 (Sigma) is added to the medium at a final concentration of 0.1% (v/v) to reduce foam formation and to minimize damage by shearing.

3.2.2. Adaptation of Insect Cells to Serum-Free and Protein-Free Medium

1. Dislodge the cells from a near-confluent monolayer by gentle pipeting.
2. Dilute 1:5 into a 50/50 mixture of serum-supplemented and serum-free medium.
3. Incubate until confluency.
4. Repeat **steps 2** and **3** once.
5. Increase the percentage serum-free medium gradually during subsequent passages (e.g., from 50% to 75% to 90% to 95% to 100%).
6. If required, transfer cells that grow in serum-free medium to suspension cultures by seeding the cells into a 250-mL spinner bottle in a starting density of at least 5.10^5 cells/mL in volumes of 50–100 mL. If not present in the medium, add Pluronic-F68 (*see* **Subheading 3.2.1.**).
7. Monitor cell growth daily; as soon as a stationary phase has been reached (usually at approximately 5.10^6 cells/mL), dilute the cells into a new spinner bottle (*see* **Note 9**).

3.2.3. Cell Culture in Bioreactors

1. Transfer cells that are in midlog phase in spinner flasks in serum-free medium (*see* **Note 10**) at a starting density of 5.10^5 cells/mL in the minimum working volume (300 mL) of a 3-L stirred bioreactor (we use bioreactors from Applikon).
2. Keep the oxygen tension at 80% of air-saturated water using pure oxygen and a porous sparger, with the oxygen flow at 5 mL oxygen/min/L medium.
3. Keep the overlay aeration at 100 mL air/min/L (volume bioreactor minus volume medium) in order to remove gaseous metabolic endproducts such as CO_2 and NH_3.
4. Agitate at 80 rpm using a marine impeller.
5. Monitor the cell density every day.
6. Add medium progressively to the maximal working volume (2.5 L for 3 L bioreactors, 5 L for a 7-L bioreactor, 10 L for a 15 L bioreactor) while maintaining a cell density of approx 1.10^6 cells/mL.
7. Infect at a density of $1.5–2.10^6$ cells/mL using a MOI of 0.01–0.1.
8. Monitor the oxygen consumption as an indication of cell growth and of progress of the infection.
9. Harvest at the maximal production level (*see* **Subheading 3.3.2.**; *see* **Note 11**).

3.3. Production of Recombinant Rhodopsin

Recombinant *his*-tagged rhodopsin is expressed as the apoprotein opsin, and is first regenerated with its ligand (11-*cis*-retinal) in a membrane environment in order to generate the more stable holoprotein. The following procedures represent the optimal conditions for production, solubilization, and purification of the holoprotein (*see* **Note 12**).

3.3.1. Infection of Insect Cells With (Recombinant) Baculovirus

1. Grow the cells to approx 80% of confluency (in flask), or to approx $1.5–2.10^6$ cells/mL (in spinner/bioreactor).
2. Infect at an MOI of 0.5–1 (in flask) or 0.01–0.1 (in spinner/bioreactor; *see* **Subheading 3.2.3.**; *see* **Note 13**).
3. Harvest at 4 d postinfection (*see* **Note 11**). If necessary, the cell pellet can be stored at −80°C.

3.3.2. Harvesting of the Infected Cells and Isolation of Cellular Membranes

1. Perform all reactions at 4°C, unless indicated otherwise.
2. Dislodge the cells from the culture flask by pipeting or with a cell scraper.
3. Collect the cells by centrifugation at 1000*g* for 10 min.
4. Suspend the cell pellet in Buffer A (*see* **Subheading 2.3.1.**) at 10^8 cells/mL.
5. Homogenize with six strokes in a motor-driven Potter-Elvehjem homogenizer at 150 rpm.
6. Centrifuge for 10 min at 40,000*g*. Discard the supernatant.

3.3.3. Regeneration and Extraction of Rhodopsin

1. Resuspend the crude membrane pellet in Buffer B (*see* **Subheading 2.3.2.**) at a concentration equivalent of 10^8 cells/mL. Starting with the next step, all experiments are performed in the dark room under dim red light using a long-pass (>630 nm) filter (Schott).
2. For regeneration of rhodopsin, add to this suspension 11-*cis*-retinal from a stock solution in dimethylformamide ***(5)*** to a final concentration of 5 nmol per 10^8 cells-equivalent and rotate for 1.5 h at room temperature under an argon atmosphere.
3. Add DoM to a final concentration of 1% (w/v), and β-mercaptoethanol to a final concentration of 5 m*M*.
4. Rotate for 1 h at 4°C under an argon atmosphere to solubilize the pigment.
5. Centrifuge for 30 min at 120,000*g* to remove aggregates and unsolubilized material. Use the supernatant for affinity purification (*see* **Subheading 3.3.4.**).

3.3.4. Purification of his-*Tagged Rhodopsin by Immobilized Nickel Affinity Chromatography*

1. Determine the yield of functional rhodopsin by recording the spectrum of an aliquot of the supernatant from 250 to 700 nm (we use a Perkin-Elmer Lambda-15 spectrophotometer). The concentration of rhodopsin is determined from the difference in absorbance at 498 nm before and after photobleaching in the presence of 50 m*M* hydroxylamine for 5 min with a 100 W Tungsten light bulb, equipped with a 430-nm long-pass filter (Schott). The molar absorbance coefficient at 498 has been determined as 40,500 $M^{-1}\cdot cm^{-1}$ (*see* **Note 12**).
2. Dilute the supernatant with Buffer C (*see* **Subheading 2.3.3.**) to the equivalent of 10^8 cells/mL. Adjust the pH of the membrane extract to pH 7.0–7.2 with 200 m*M* *bis*-trispropane. This pH is the best compromise between maximal stability of rhodopsin (pH 6.5) and maximal binding potential of the Ni^{2+}-matrix (pH 7.5–8.0).
3. Wash the required amount of Ni^{2+}-nitriloacetic acid resin (Qiagen; 10 μL of packed gel per nmol rhodopsin) with 10 volumes of destilled water, followed with 10 volumes of Buffer A (*see* **Note 14**).
4. Apply the diluted supernatant to the column at a flow rate of 1 mL/h/mL gel and wash with 10–15 column volumes of Buffer A, until no more protein is detected by measuring the A_{280} of the flowthrough.
5. Wash with two column volumes of a linear gradient from 1–5 mM histidine in Buffer D. This will remove contaminating proteins with low affinity for the Ni^{2+} matrix such as cytochromes.
6. Elute with approx 2 column volumes of Buffer E (*see* **Subheading 2.3.3.**), screen the fractions with UV-VIS spectroscopy, and combine the active fractions (A_{500} > 0.02). The purification of *his*-tagged rhodopsin as monitored by SDS-PAGE and by spectral analysis are shown in **Figs. 2** and **3**, respectively (*see* **Note 15**).
7. Proceed immediately with the reconstitution (*see* **Subheading 3.3.5.**), because the purified protein has a low stability under these experimental conditions.

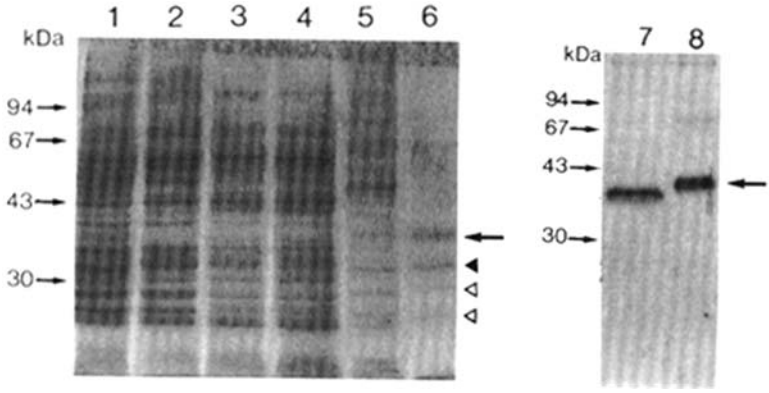

Fig. 2. Purification profile of recombinant *his*-tagged rhodopsin using a Ni^{2+}-affinity column analyzed by PAGE and protein staining. Fractions were analyzed by SDS-PAGE followed by protein staining with Coomassie blue. 1: Sf9 cell extract that was loaded on the column; 2: nonbound fraction; 3, 4: fractions eluted upon washing with buffer D; 5, 6: fractions eluted with Buffer E. The positions of the glycosylated and non-glycosylated forms of rhodopsin are indicated with a large arrow and a small filled arrowhead, respectively. Note the minor contaminants in the eluted fraction (lane 6; indicated with open arrowheads). These are no longer detectable in the reconstituted pigment (lane 8). Also note the *His*6 tag-induced difference in migration rate between native rhodopsin (lane 7) and recombinant, *his*-tagged rhodopsin (lane 8).

3.3.5. Reconstitution of Rhodopsin with Retina Lipids

Determination of functional characteristics is performed after reconstitution with lipids, because a lipid bilayer mimicks the native membrane environment, and consequently provides the best preservation of thermal stability and functional integrity (*see* **Note 16**).

1. Combine the purified fractions and add an approximately 100-fold molar excess of bovine retina lipid extract ***(6)*** in Buffer B (*see* **Subheading 2.3.2.**).
2. Extract the detergent from the mixed proteolipid/detergent micelles by adding solid β cyclodextrin ([heptakis 2,6-di-O-methyl]-β-cyclodextrin; Sigma) to a final concentration of 25 mM ***(7)***. Formation of proteoliposomes can be monitored as a pronounced increase in light scattering, e.g., measured as an increase in absorbance over the range 500–700 nm.
3. Purify the proteoliposomes by layering the mixture on a sucrose step gradient (10, 20, 45% sucrose (w/w) in Buffer B, and centrifuge for 16 h at 200,000*g*.
4. Isolate the proteoliposome fraction from the 20–45% interphase, dilute with four volumes of distilled water and pellet by centrifugation for 30 min at 200,000*g*.
5. Store the membrane pellets in light-tight containers at −80°C.

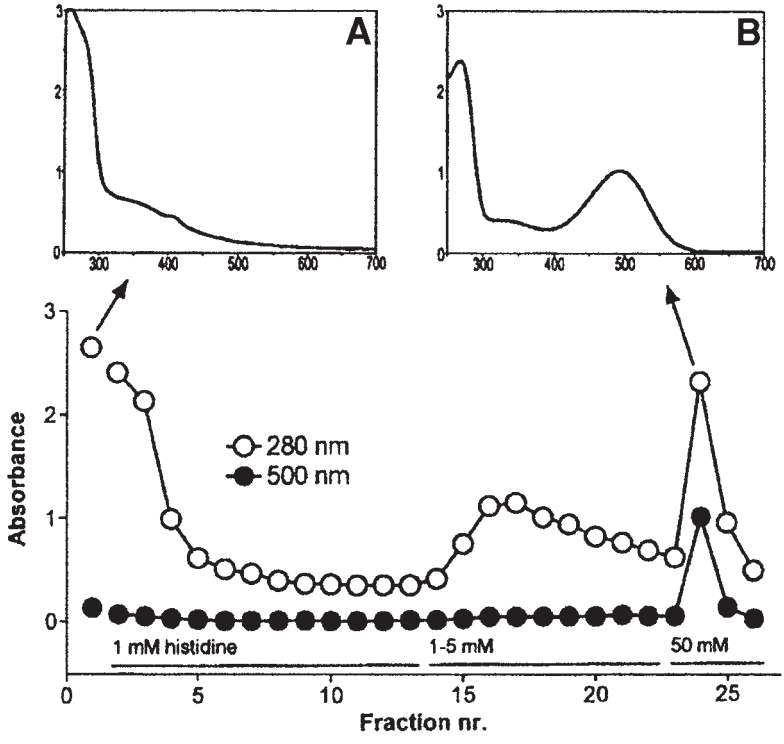

Fig. 3. Purification profile of recombinant *his*-tagged rhodopsin using a Ni^{2+}-affinity column monitored by UV-visible spectroscopy. Fractions were analyzed by measuring their spectral characteristics. (**A**) Spectrum of the total membrane extract that was loaded on the column; (**B**) spectrum of the major fraction that was eluted with 50 m*M* histidine. This shows the characteristic absorbance spectrum of rhodopsin. The abscissa presents wavelength in nm and the ordinate presents absorbance in arbitrary OD units.

3.4. Functional Analysis of Rhodopsin

Ligand binding ("regeneration" in case of the visual pigments; *see* **Subheading 3.3.3.**) and ligand affinity represent important functional parameters for any receptor. The other most relevant functional parameter for G protein-coupled receptors is binding and activation of its cognate G protein. Here we describe the G protein (transducin) activation assay we use for the analysis of rhodopsin (***8***; *see* **Note 17**). In the case of visual pigments, time-resolved spectroscopical studies of the photocascade also present useful indicators of functionality, and FT-IR studies provide structural information, that has high functional relevance as well. For further information we refer to comprehensive reviews ***(9,10)***.

3.4.1. Transducin Activation Assay

1. Illuminate the transducin stock in the presence of 10 m*M* hydroxylamine on ice for 30 min with a 100 W Tungsten light bulb behind a KG-1 filter, in order to quench any background photoactivity caused by impurities, left during the transducin extraction from bovine eyes (***8***; *see* **Note 17**).
2. Combine rhodopsin (final concentration 5 n*M*) and transducin (approx 100 n*M*) in Buffer F (*see* **Subheading 2.4.1.**).
3. Activate the rhodopsin by illumination for 5 min with a 100 W Tungsten light bulb behind a KG-1 heat filter (Schott) at room temperature.
4. Pass the solution through a 0.2-μ*M* filter in order to reduce background scattering.
5. Transfer the reaction mixture to to a quartz cuvette containing a magnetic stirring bar.
6. Measure the fluorescence on a spectrofluorometer at room temperature with constant stirring (excitation at 295 nm / bandwidth 1.5 nm; emission at 337 / bandwidth 15 nm; we use a Shimadzu RF-5301 spectrofluorometer) with a recording speed of one data point per second.
7. Record for 200 s in order to establish a baseline.
8. Add guanosine 5'-[γ-thio]triphosphate (GTPγS) to a final concentration of 2.5 μM and record for at least another 600 s. The activation of transducin is measured as the increase in emission intensity (*see* **Note 17**).
9. Repeat the reaction with 5 n*M* opsin for the determination of background activity. Opsin is prepared by illuminating a concentrated rhodopsin suspension in the presence of 50 m*M* hydroxylamine (10 min, 100 W light bulb, OG530 long-pass filter).
10. The initial rate of increase in the fluorescence intensity during the first, linear part of the curve represents the initial G protein activation rate, and is a good indicator for the signalling capacity of the pigment (a typical example is shown in **Fig. 4**). We refer for a more complex treatment of the data to extract rate constants to ***(10,11)***.

4. Notes

1. For detailed structural and functional studies, expression of GPCRs as recombinant proteins is a *sine qua non*, as only heterologous systems allow the production of proteins with purification tags, targeted mutations, and/or stable isotope-labeled amino acids in sufficient quantities.
2. Although the insect cell is capable of complex glycosylation, baculovirus infection results in partially processed high-mannose forms, depending on the time period post infection. Nevertheless, the majority of the proteins expressed in the baculovirus system shows a biological activity that is identical to that of fully processed, native proteins. Mutations that affect correct folding, membrane insertion, and/or translocation of rhodopsin to the plasma membrane usually manifest themselves in defective glycosylation and reduced ligand binding (regeneration).
3. Most commercial vectors employ the very strong, late-phase polyhedrin promoter to control expression. This is the promoter we also use routinely. We did obtain comparable expression levels with the late P10 promoter, but the use of the early

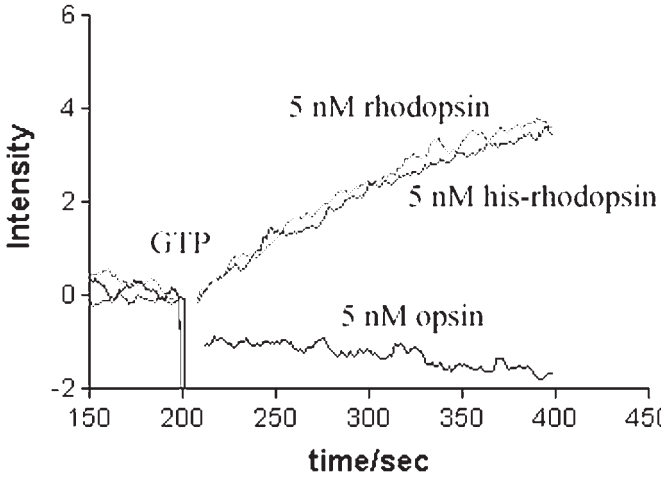

Fig. 4. G protein activation assay of rhodopsin. Activation of the G protein transducin was assayed by the increase of Gα tryptophan fluorescence upon binding of GTPγS. Bovine rhodopsin, recombinant *His*-tagged rhodopsin (*his*-rhodopsin), and bovine opsin were mixed with transducin and illuminated. After stabilization of the fluorescence emission intensity, GTPγS was added (GTP). The rate of the subsequent increase in emission intensity represents the capacity of rhodopsin to activate transducin. The emission level was normalized to zero before the addition of GTPγS. It is obvious that the *his*-tag does not significantly influence the activity of rhodopsin. The apoprotein opsin has very little residual activity. The gap in the curve is caused by opening and closing of the lid of the spectrofluorometer, necessary for the addition of GTPγS.

Basic Protein promoter was not successful ***(12)***. In our experience, the baculovirus has no clear codon preference, although the base composition around the start codon may have some influence ***(13,14)***. Large (*His*10) as well as smaller (*His*6) tags will result in a modest reduction of the expression level of up to 30%.

4. A low concentration of EDTA is present in order to complex heavy metal cations with pro-oxidant activity, for which EDTA has a high affinity.
5. The total yield of recombinant protein can be determined by ELISA ***(15)*** or estimated by immunoblot using a concentration range. For most purposes, however, the yield of functional protein is the most important parameter. The fraction of functional over total protein may vary between 80% (rhodopsin) and 30% (e.g. cone pigments). For GPCRs the functional yield is best assayed by ligand binding (*see* **Subheading 3.3.3., Subheading 3.3.4.**).
6. In the two most commonly used procedures recombinant virus is generated by: 1) homologous recombination after cotransfection of Sf9 cells with fragmented viral DNA and a transfer vector containing a plasmid for bacterial replication, a viral promoter and flanking sequences, and the cDNA insert; 2) site-specific transposi-

tion with Tn7 to insert cDNA into bacmid DNA that is propagated in *E. coli*. The major advantage of the latter system is the easy maintenance of the viral genome as bacmid DNA in *E. coli*.

7. We observed no significant differences in the yield of functional rhodopsin per cell between the Sf9 cell line and other cell lines such as the Sf21 cell line, the *Trichoplusia ni* BTI-TN5B1-4 (High five) cell line, and a *Mamestra brassicae*-derived cell line [unpublished results and ***(2)***]. We routinely use the Sf9 cell line since, in our hands, it performs best in suspension culture.
8. We found that Sf9 cells adapted to serum-free medium (InsectXpress, BioWhittaker; *see* **Subheading 3.2.2.**) can be propagated as a monolayer for at least 6 mo. These adapted cells reach a higher cell density at confluency than cells growing in TNM-FH supplemented with 10% fetal calf serum.
9. After propagating Sf9 cells in spinner cultures for a period of approx 3 mo while diluting the cells twice a week, they start to grow slower, to fragment more easily, and to produce less recombinant protein. At that time, we thaw a new batch of cells from a liquid nitrogen stock and, if necessary, restart the procedure to adapt the cells to serum- and protein-free medium.
10. In bioreactors, we prefer to use serum-free and protein-free medium mostly to reduce the costs, but also to facilitate the labeling of recombinant proteins with stable isotope-containing amino acids. For the latter, we refer to ***(3)***.
11. The optimal MOI and especially the number of DPI before the cells are harvested should be determined for every type of protein individually, as the yield of functional protein may differ considerably between proteins and the baculovirus promoters that are used. We describe here the data obtained with the polyhedrin promoter.
12. Functional expression levels vary depending on the type of receptor over a range of 2–50 pmol/10^6 cells. For rhodopsin, a functional expression level in the range of 10–20 pmol/10^6 cells (6–12.10^6 copies/cell) is acceptable. For cone pigments functional yields may vary between 4–10 pmol/10^6 cells.
13. In order to monitor the capacity of fresh cells or new recombinant virus for expression of recombinant protein, we routinely use cells growing in monolayers and an MOI of at least 1. Under these conditions production levels usually peak at 3 DPI. Lower MOIs are used for recombinant protein production in spinner flasks (0.05–0.5) or bioreactors (0.01–0.1). In the latter conditions, production levels should be checked over a period of 4–7 DPI. In fact, low MOI's may result in a higher production per unit volume (***4***; *see* **Fig. 1**).
14. Although a N-terminal and a C-terminal hexahistidine-tagged rhodopsin are equally functional, we have found no condition in which N-terminally *His*-tagged rhodopsin bound to the immobilized metal ***(4)***. A more stable binding to the column is obtained with a *His*10, although we usually see a higher cellular production using a *His*6 tag. Results depend on the type and make of the matrix (unpublished data). For a maximal recovery of functional protein, the type of detergent(s) and its concentration should also be determined for each membrane protein individually. Criteria are the thermal stability of the pigment in the solubilized state and the affinity of the pigment-detergent complex for the affinity

matrix. In this respect, DoM is a good choice for rhodopsin, whereas for cone visual pigments good results may be obtained with a mixture of 0.5% CHAPS (Sigma) and 0.5% DoM ***(12,16)***.

15. At this stage the purity varies between 60–80% and minor contaminants are detectable, that are largely removed during reconstitution (*see* **Fig. 2**). The purity can also be estimated from the A_{280}/A_{500} ratio. This ratio is 1.7 ± 0.1 for highly purified rhodopsin.
16. We have observed that, instead of a natural lipid mixture, the commercially available soybean extract asolectin (Sigma) also produces fully functional proteoliposomes with many receptors at a molar lipid to protein ratio of 100–150.
17. Activation of transducin results in binding of GTP to its α-subunit with a concomitant increase in fluorescence of a nearby tryptophan residue, that is employed for assay purposes ***(17)***. Instead of GTP we use GTPγS, that after binding cannot be hydrolysed by the intrinsic GTP-ase activity of the a-subunit ***(17,18)***. A crude transducin preparation is obtained by a hypotonic wash of isolated bovine rod outer segments ***(19)***. Such preparations contain 80 ± 10% transducin (Tα,β,γ) on a protein basis. This suffices for this activation assay, provided that background activity is eliminated by preillumination (*see* **step 1**). The transducin content in such a preparation is estimated from a protein determination (Biorad), taking the transducin proportion as 80% and 86 kDa as the molecular weight for Tα,β,γ.

    Constitutive activity of the pigment can be assayed in the dark by a modification of the here described transducin activation procedure ***(20)***.

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8

Preparation of Glycerol Facilitator for Protein Structure and Folding Studies in Solution

Darren Manley and Joe D. O'Neil

1. Introduction

Progress in the elucidation of protein folding pathways is contributing to a deeper understanding of the information encoded in genomes ***(1)***. However, the study of membrane protein folding lags behind similar studies of water-soluble proteins ***(2)***. Some of the impediments include poor expression of membrane proteins possibly caused by their misfolding, aggregation, improper transport, and insertion into membranes, or their toxicity when overexpressed ***(3)***. In vitro, poor solubility properties lead to difficulties in purification, refolding, crystallization, and nuclear magnetic resonance analysis. The *Escherichia coli* glycerol facilitator (GF) is an attractive model for studying membrane protein folding ***(4–6)***. *E. coli* is by far the most successful and versatile vehicle for the production of recombinant proteins and by expressing a native bacterial protein in *E. coli* difficulties associated with the expression of foreign genes, such as rare codon usage ***(7)*** and posttranslational modifications, are avoided. High levels of GF protein are unlikely to be toxic to the cell; the facilitator is highly specific for glycerol transport ***(5,6)*** and glycerol can be omitted from the growth medium. Uncontrolled water transport might be problematic owing to osmotic pressure effects, however, the facilitator does not transport water nearly as efficiently as the aquaporins, and aquaporin-1 is naturally highly expressed in red blood cells ***(5,6)***. In addition, it seems unlikely that high levels of intracellular glycerol would be toxic to *E. coli* and in indeed we find maximum expression using the glycerol-rich medium Terrific Broth (TB) ***(8)***.

The glycerol facilitator expression system described here uses a commercially available vector that places the *glpF* gene under the control of the T7 pro-

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moter ***(9)***. A major advantage is that it permits the use of rifampicin to inhibit the *E. coli* RNA polymerase (but not the T7 RNA polymerase), thereby halting bacterial protein synthesis permitting the bacterium to use all available resources for recombinant protein synthesis ***(10,11)***. In our hands, this results in a two–threefold increase in the yield of glycerol facilitator compared to expression in the absence of rifampicin ***(4)***. The *E. coli* strains BL21(DE3) and BL21(DE3)pLysS ***(9)*** can both be used as protein expression hosts for glycerol facilitator however the pLysS-containing cells express small amounts of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. This is useful for reducing basal protein expression in the absence of inducer and has been reported to improve the viability of cells expressing toxic proteins ***(3,12)***. The pLysS strains enjoy an additional convenience because T7 lysozyme accelerates cell lysis by hydrolysing *E. coli* cell wall peptidoglycan, which it can access after the cells have been frozen and thawed.

The recombinant glycerol facilitator described here incorporates an amino-terminal His_6 tag for rapid protein purification by immobilized metal affinity chromatography ***(13,14)*** that works just as well for the purification of membrane proteins solubilized in detergent solutions as for water-soluble proteins. The protein also contains a thrombin cleavage site for removal of the His_6 segment and an 11-residue T7-epitope for Western immunodetection. Up to 7 mg of pure recombinant GF are produced per liter of cell culture, the protein is soluble in a variety of detergents suitable for spectroscopic analysis in concentrations up to 90 μM, and is stable over a period of weeks ***(4)***.

2. Materials

1. (PCR) Polymerase chain reaction primers are made in the Department of Microbiology, University of Manitoba.
2. DNA amplification is performed using the Expand High Fidelity PCR system (Boehringer Mannheim, Laval, QC, Canada).
3. Prep-A-Gene DNA purification kit is purchased from Bio-Rad Laboratories (Mississauga, ON).
4. Mutagenesis primers, restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of DNA polymerase I are purchased from Life Technologies (Rockville, MD).
5. Helper phage R408 is obtained from Promega (Madison, WI).
6. Ready-to-go pUC18, pre-cut at the SmaI site, is from Pharmacia Biotech (Baie d'Urfé, PQ).
7. The pET28b(+) expression vector, anti-T7 antibody-alkaline phosphatase conjugate, and the bacterial strains Novablue, BL21(DE3), and BL21(DE3)pLysS are obtained from Novagen (Madison, WI).
8. Nickel-nitrilotriacetic acid (NTA) resin is from Qiagen (Toronto, ON).

9. Sodium dodecyl sulphate (SDS), octyl-β-D-glucopyranoside (OG), rifampicin, diatomaceous earth, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate are obtained from Sigma (St. Louis, MO).
10. Deoxynucleotidetriphosphates are from Boehringer Mannheim (Laval, QC) that was also the source of the 5-bromo-4-chloro-3-indolyl-β–D-galactopyranoside (BCIG).
11. Suspension solution: 50 m*M* Tris-HCl pH 8.0, 10 m*M* ethylene diamine tetraacetic acid (EDTA), and 100 μg/mL Deoxyribonuclease-free Ribonuclease A.
12. 0.2 *M* NaOH containing 1% SDS.
13. 4 *M* potassium-acetate, pH 4.8.
14. 6 *M* guanidine hydrochloride.
15. Wash buffer: 20 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.4 *M* NaCl, 50% ethanol.
16. TE buffer: 20 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
17. 1 m*M* IPTG.
18. Buffer A: 50 m*M* sodium phosphate pH 7.6, 50 m*M* NaCl, and 10 m*M* β-mercaptoethanol.
19. Buffer A with 150 m*M* SDS, pH 7.6.
20. Buffer A with 150 m*M* SDS, pH 7.2.
21. Buffer A with 150 m*M* SDS, pH 6.5.
22. Buffer A with 3 m*M* dodecyl-β-D-maltoside (DM; Anatrace, Maumee, OH), pH 7.6.
23. Buffer A with 3 m*M* DM, pH 6.5.
24. Buffer A with 30 m*M* DM, pH 4.0.
25. Buffer A with 250 m*M* xylitol.

3. Methods

3.1. Expression Vector Construction.

This section outlines the strategy used in the construction of the expression plasmid and gives references where standard molecular biological protocols are used. The *glp*F gene can be spliced into pET28b(+) ***(14)*** as follows: *GlpF* DNA is amplified from *E. coli* genomic DNA ***(15)*** using the PCR ***(16)*** and the Expand High Fidelity PCR system from Boehringer Mannheim. The forward PCR primer oligonucleotide (5'CATTAACTCTTCAG**GGATCC**GATTATGAGTC3') is identical in sequence to residues 188–218 of the published sequence of the *glpF* gene ***(17)*** (*see* **Fig. 1**) and its upstream region that encodes a naturally occurring *BamHI* restriction site (shown above in bold type). The reverse primer is complimentary to residues 1043–1081 of *glpF* and its downstream region except that a noncomplimentary *XhoI* restriction site (bold) is incorporated between residues 1070–1075 (5'ATGTTT**CTCGAG**CCCGTAGTCATATT ACAGCGAAGCTTT3'). The amplified DNA is purified by agarose gel electrophoresis ***(18)***, the *glpF* DNA band is excised, and the DNA is extracted from the agarose using the Prep-A-Gene DNA purification kit ***(19)***. The 3' adenine

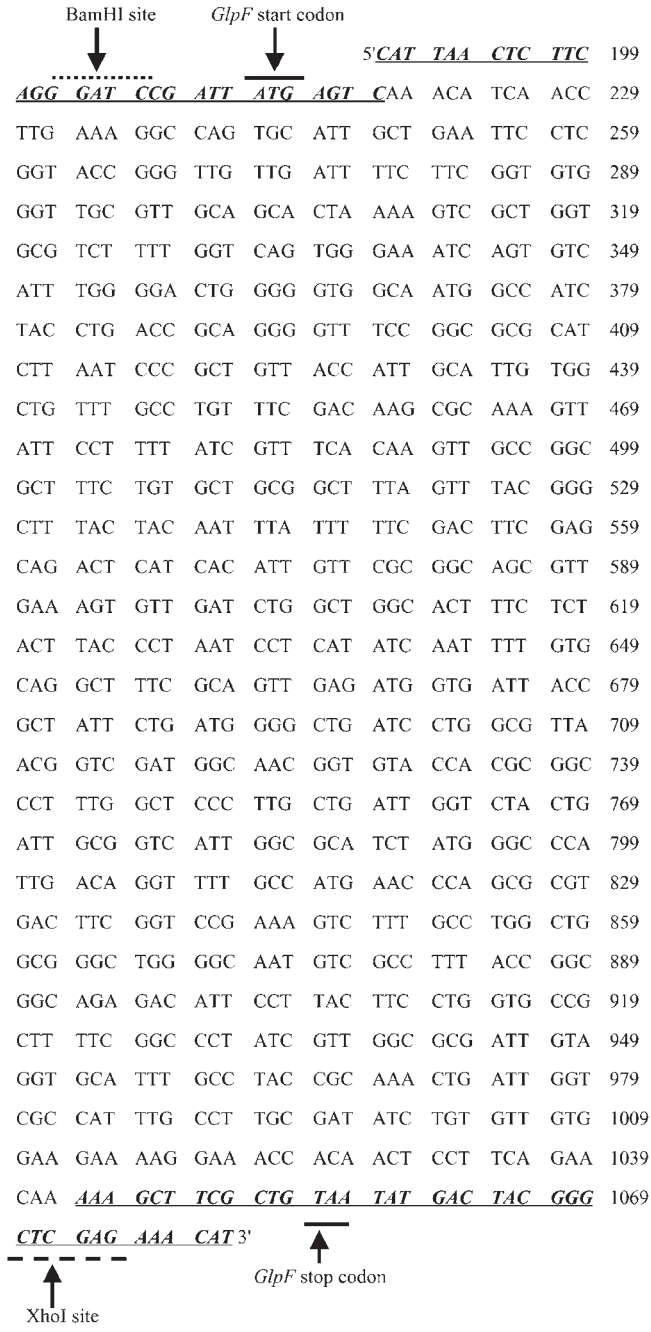

Fig. 1. The expected DNA sequence of the *glpF* PCR product based on the sequence of the gene ***(17)*** and the introduction of an *XhoI* restriction site. The *glpF* start and stop codons and the restriction sites used for cloning purposes are indicated. Also indicated are the locations of the forward and reverse PCR primers (bold, italic, underline).

overhangs, introduced by *Taq* DNA polymerase, are filled in by the Klenow fragment of DNA polymerase I ***(20)*** to produce a DNA fragment with two blunt ends. The 5'DNA termini are phosphorylated with T4 polynucleotidekinase ***(18)*** and the DNA is inserted into the blunt-end *SmaI* site of pUC18 using T4 DNA ligase ***(18)***. The resultant plasmid is designated pUC18-*glpF*.

Competent Novablue cells are transformed with pUC18-*glpF* ***(12)*** and transformants are selected (*see* **Note 1**) by growth on plates containing LB (Luria-Bertani) medium ***(18)*** supplemented with 12.5 μg/mL tetracycline, 25 μg/mL ampicillin, 30 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG), and 30 μg/mL isopropylthio-β-D-galactoside (IPTG). Plasmids containing *glpF* DNA are purified using the Prep-A-Gene purification kit as described below ***(19)***. The DNA encoding the glycerol facilitator is excised from pUC18 using the *BamHI* and *XhoI* restriction sites. The DNA is purified by agarose gel electrophoresis, recovered using the Prep-A-Gene kit, and inserted into a similarly digested pET28b(+) expression plasmid. The resulting construct is designated pET28*glpF*. The *E. coli* strains BL21(DE3) and BL21(DE3)pLysS ***(9)*** can both be used as protein expression hosts. Transformants containing the pET28*glpF* construct are selected on LB-agar plates supplemented with 34 μg/mL chloramphenicol and 30 μg/mL kanamycin.

3.1.1. DNA Isolation and Sequencing

Highly pure plasmid DNA is rapidly isolated from 2 mL *E. coli* cultures using a method adapted from that of Kim and Pallaghy ***(22)***.

1. Cells from an overnight culture are chilled on ice for 5 min, centrifuged at 4000*g* for 10 min at 4°C and resuspended in 500 μL of suspension solution.
2. The cells are lysed by addition of 500 μL of 0.2 *M* NaOH containing 1% SDS.
3. After incubation for 5 min at room temperature the mixture is neutralized with the addition of 500 μL of 4 *M* potassium-acetate, pH 4.8.
4. Precipitated protein, genomic DNA, and cellular debris are removed by centrifugation on a microcentrifuge (12,700*g*, 5 min) and the DNA-containing supernatant is transferred to a microcentrifuge tube containing an equal volume of diatomaceous earth suspension (*see* **Note 2**).
5. An equal volume of 6 *M* guanidine hydrochloride is added to the DNA/diatomaceous earth mixture, it is mixed gently, and centrifuged; the supernatant is carefully removed and discarded.
6. The guanidine wash is repeated twice (*see* **Note 3**) followed by three washes with Wash buffer.
7. The pellet is air-dried and the pure plasmid DNA is eluted by resuspending the pellet in 100–200 μl of water or TE buffer followed by removal of the diatomaceous earth by centrifugation and retention of the supernatant. The purity and yield of the DNA is checked by agarose gel electrophoresis ***(23)***, and then is stored at −20°C.

All plasmids containing the *glpF* gene should be checked by restriction mapping using the *BamHI* and *XhoI* enzymes, and DNA sequencing. DNA is prepared for sequencing by precipitation with polyethylene glycol using a modified alkaline lysis method suggested by Perkin-Elmer (http://www.ucalgary.ca/~dnalab/Plasmidprep.html; *see also* **ref. *24***). We sequenced the *glpF* DNA using a commercial service and two PCR-induced mutations, Asp168Val (GAC→GTC) and a silent mutation for valine-146 (GTG→GTA), were discovered. The D168V mutation was reverted to wild type by Kunkel mutagenesis ***(25)*** using f1 helper phage R408 and the f1 origin of replication on the pET28b(+) vector to produce the single-stranded plasmid DNA ***(26,27***).

3.2. Glycerol Facilitator Overexpression and Purification

Here, we describe the preparation of glycerol facilitator dissolved in SDS and dodecyl-β-D-maltoside (DM). We have used similar procedures to prepare protein in octyl-β-D-glucopyranoside (OG).

1. BL21(DE3) pLysS cells containing pET28*glpF* are grown in antibiotic-supplemented (34 μg/mL chloramphenicol and 30 μg/mL kanamycin) TB ***(8)*** at 37°C.
2. Glycerol Facilitator expression is induced with the addition of 1 m*M* IPTG when the culture reaches an optical density at 600 nm (OD_{600}) of 0.6–0.7.
3. The bacterial RNA polymerase is arrested by the addition of rifampicin (200 μg/mL) at 1 h following induction and the cells are harvested after a further 2 h of growth. The cell cultures are chilled on ice for 5 min and collected by centrifugation.
4. The cell pellets are flash frozen in liquid nitrogen and, if necessary, stored at –20°C.
5. The cell pellets are thawed, resuspended in Buffer A, and lysed by passage through 18- and 22-gage needles.
6. DNA is sheared by sonicating (Fisher Sonic Dismembrator Model 300) the lysed cells on ice three times for 30 s at 30% power.
7. The ruptured cells are centrifuged at 4°C for 20 min at 2800*g*.
8. The resulting pellet is resuspended in about 100 mL of a Buffer A-detergent solution per liter of cell culture, and stirred at room temperature for 1 h. The concentrations of the detergents are 150 m*M* SDS or 3 m*M* DM. For solubilization with DM it is necessary to extend the incubation time a further 30 min, or until the majority of the visible cellular material has dissolved. Alternatively, a higher concentration of DM may be used.
9. For SDS-solubilized cells, centrifugation is performed at room temperature using a Dynac bench top centrifuge for 20 min at 1100 g. In the case of DM-solubilized cells, insoluble material is removed by centrifugation at 4°C.
10. The supernatant is added to 1 mL of settled Ni^{2+}-NTA Sepharose resin preequilibrated in a Buffer A-detergent solution, and the slurry is stirred gently at room temperature for 1 h.
11. Prior to being poured into a glass column the Ni^{2+}-NTA Sepharose is separated from excess buffer by low speed centrifugation for 5 min at 500*g*.

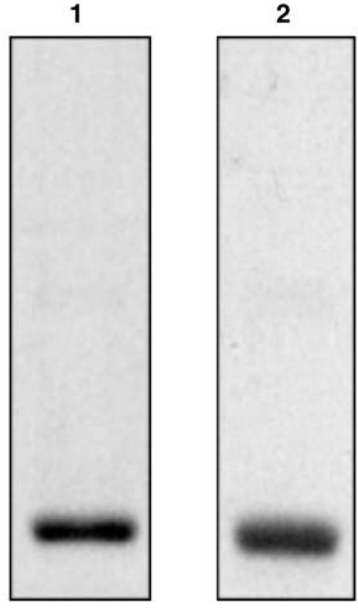

Fig. 2. Coomassie-stained SDS-PAGE electrophoregrams of glycerol facilitator purified in: lane 1, 150 m*M* SDS; lane 2, 30 m*M* DM.

12. For the preparation of protein in SDS, the resin is then washed with several volumes of Buffer A plus 150 m*M* SDS at pH 7.6, followed by a second wash at pH 7.2. For the DM preparation the column is washed in several column volumes of Buffer A plus 3 m*M* DM at pH 6.5. Elution is begun once the A_{280} is below 0.1.
13. Glycerol Facilitator is eluted in Buffer A plus 150 m*M* SDS at pH 6.5 (*see* **Note 4**) or 30 m*M* DM at pH 4.0. One ml fractions are collected and analyzed by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ***(29,30)*** and Western immunoblotting ***(29)*** techniques (*see* **Note 5**).

Fig. 2 shows Coomassie-stained electrophoregrams of SDS- and DM-purified glycerol facilitator. Although only monomeric protein is observed in the Coomassie-stained gels, Western blots (not shown) indicate that small amounts of dimer, trimer, and tetramer are invariably present ***(4)***. The expected molecular weight (M_r) of the expressed glycerol facilitator including the N-terminal fusion tags is 33,505 kDa and its predicted isoelectric point (p*I*) is 7.56 (calculated using the program MacVector 6.5, designed by Oxford Molecular).

3.3. Xylitol Transport Assay

The recombinant glycerol facilitator inserts into the bacterial inner membrane and is functional ***(4)***. However, glycerol is transported with a half time of

less than 10s and this necessitates the use of rapid kinetic methods, such as stopped-flow, for measurement ***(32)***. An alternative exploits the fact that the glycerol facilitator transports xylitol more slowly than glycerol and this permits functionality to be ascertained with the use of a spectrophotometer and manual mixing ***(33)***. Water and solute transport cause changes in the volume and shape of *E. coli* that are detectable as changes in turbidity. The theory behind these effects is clearly explained in a review by Koch ***(34)***.

1. Induced and noninduced *E. coli* are harvested by centrifugation at 4°C for 10 min at 2800*g*.
2. The cell pellet is resuspended in Buffer A at a volume equal to one third of the original cell culture.
3. The concentrated cells are then diluted into Buffer A containing 0 or 250 m*M* xylitol giving a final OD_{600} of about 1.0. Dilution into xylitol results in an immediate increase in turbidity (OD_{600}) owing to cell shrinkage from the sudden increase in external osmotic pressure; this is followed by a slower cell swelling and drop in turbidity that is complete within 5 min. The noninduced cells, lacking glycerol facilitator, initially shrink but cannot transport glycerol and do not re-swell.

3.4. Circular Dichroism

Circular Dichroism (CD) spectropolarimetry can be used to quantify secondary structure in proteins, detect the presence of tertiary structure, and follow structural changes during protein folding and unfolding. We acquire CD spectra with a Jasco-500A CD spectropolarimeter interfaced to a personal computer via an analog-digital converter. Sample temperature is controlled by a Haake G circulating water bath. For far ultraviolet (UV) CD experiments, GF is eluted from the Ni^{2+}NTA column as aforementioned except that the buffer is 10 m*M* sodium phosphate containing 10 m*M* NaCl and detergent (150 m*M* SDS at pH 6.5 or 30 m*M* DM at pH 4.0 or 100 m*M* OG at pH 4.0). If required, the pH of the eluted protein can be adjusted by dilution with detergent-buffer solutions at the appropriate pH. Usually, several fractions collected from the column are at the appropriate concentration (6–10 μ*M*) for far UV CD analysis. CD spectra of purified GF are acquired utilizing the CD optimization parameters of Hennessey and Johnson ***(35)***. It is imperative that the absorbance properties of all buffer components, including detergent, be measured before attempting to measure CD spectra on protein solutions. Total absorbance, including the cell, solvent, and protein, is kept below 1.0 above 200 nm so that at least 10% of the light is transmitted above 200 nm ***(36)***. The samples are placed in a water-jacketed quartz cuvet with a 0.05-cm path length and maintained at 25°C. Nitrogen gas is used to flush oxygen from both the optics of the spectropolarimeter and the sample chamber and is applied at a flow rate of 5 L/min. CD spectra are collected at 2 nm/min between 260–205 nm with a time

constant of 8. Below 200 nm, atmospheric oxygen absorption increases. To increase the number of photons collected at lower wavelengths the N_2 (g) flow rate is increased to 25 L/min and spectra are collected at 0.5 nm/min with a time constant of 32 between 210–185 nm. Baselines are collected in the same fashion on detergent-buffer solutions and spectra are baseline corrected. The two portions of the far UV spectra are digitally combined using the overlap region between 210–205 nm.

Far UV CD spectral analysis requires precise determination of protein concentration. Recombinant glycerol facilitator concentration is determined using the calculated molar absorptivity of 38,305 $M^{-1}\cdot cm^{-1}$ at 280 nm. Ellipticity (θ) is calculated using the equation: $\theta = m^\circ\ (mV \cdot C)$ where m° is the sensitivity setting of the spectropolarimeter in millidegrees per cm, mV is the baseline-corrected signal in millivolts, and C is an experimentally determined conversion factor (*see* **Note 6**). Mean Residue Ellipticity ($[\theta MRE] \times 10^{-3}$ deg · cm^2 · $dmole^{-1}$) is calculated using the equation: $[\theta MRE] = \{(M_r / n) \cdot \theta\}\ \{(10 \cdot R) \cdot c\}$ where M_r is 33,505 grams per mole, n is 315 peptide bonds, θ is the measured ellipticity in millidegrees, l is the cell pathlength (0.05 cm), and c is the protein concentration in g/L. Deconvolution of the far-UV CD spectra into pure component spectra can be done with a variety of available algorithms. The Convex Constraint Algorithm written by Perczel *et al.* ***(37)*** contains a basis set of CD spectra of integral membrane proteins. **Fig. 3** shows typical far UV CD spectra for glycerol facilitator dissolved in SDS and DM. The protein contains slightly higher helix content in DM than in SDS ***(4)***.

Aromatic ellipticity is significantly weaker than peptide bond ellipticity and this necessitates the preparation of more concentrated protein solutions. We reasoned that elution of concentrated glycerol facilitator would result from a rapid reduction in the pH of the Ni^{2+}-NTA resin. For near-UV analysis, protein was prepared as aforementioned except that in the case of SDS-solubilized protein elution was done with SDS-containing Buffer A at pH 5.0. Before applying the elution buffer to the resin, the wash buffer is drained from the column. This permits the elution of protein in detergent at concentrations between 50 and 90 μ*M*. For the acquisition of near UV CD spectra the concentrated protein is placed in a cylindrical quartz cuvette with a 0.5-cm pathlength and spectra are collected at 2 nm/min between 320–260 nm with a time constant of 8 s.

4. Notes

1. pUC vectors carry the amino-terminal fragment of the β-galactosidase gene. Upon expression in the presence of IPTG, the pUC-encoded fragment complements the defective β-galactosidase encoded by the host cell (α-complementation). Bacteria expressing both fragments of the enzyme form blue colonies in the presence of BCIG. Successful insertion of *glpF* DNA into the *SmaI* site of pUC18 disrupts the

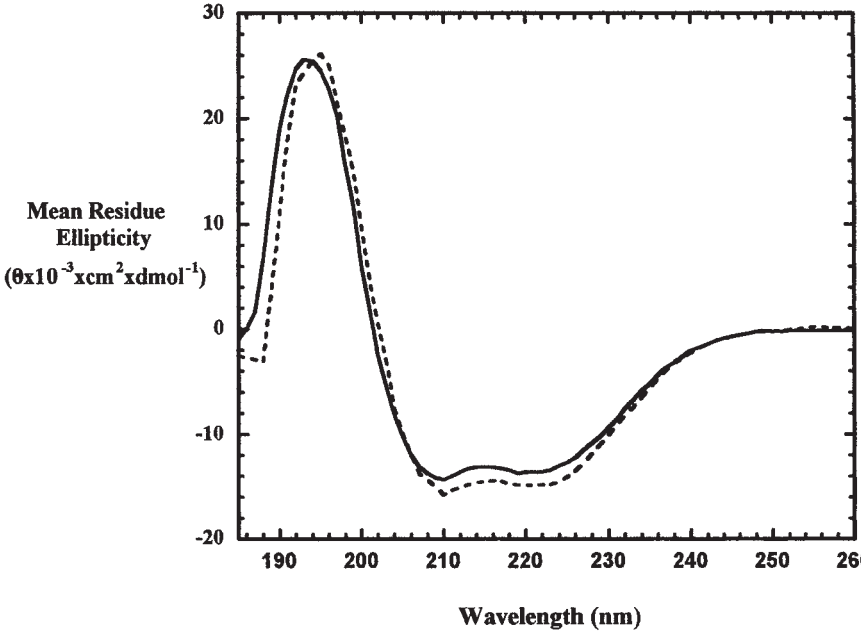

Fig. 3. CD spectra of glycerol facilitator solubilized in 10 m*M* sodium phosphate buffer containing 10 m*M* sodium chloride and detergent at 25°C. (——) GF dissolved in 150 m*M* SDS at pH 7.6; (– – –) GF dissolved in 30 m*M* DM at pH 7.0.

amino-terminal portion of the β-galactosidase gene and results in the production of white colonies ***(18)***.

2. The diatomaceous earth is prepared by suspension in double deionized water at 50 mg/mL followed by sedimentation for 1 d. The water and milky suspension above the sediment is carefully removed, replaced with an equal volume of fresh water, and the sediment is resuspended. This process is repeated until the water above the sediment remains clear and suspension-free, at which point the diatomaceous earth solution is ready for use ***(22)***.
3. Before proceeding to the next wash, the purity of the plasmid can be checked by agarose gel electrophoresis ***(23)***. The presence of low molecular weight impurities may indicate that up to three further guanidine washes are required.
4. Glycerol facilitator elutes at a higher pH in SDS presumably because the local pH at the micelle surface is lower than that measured in the bulk solution by the pH electrode. SDS micelles are known to condense protons near their surfaces ***(28)***. The protein can also be prepared in OG by elution in buffer A plus 100 m*M* OG at pH 4.0. 10 m*M* acetate can be added to the elution buffers to improve buffering at pH 4.0 however this is not necessary and is avoided in the preparation of samples for CD as it elevates the optical density of the solutions especially at lower wavelengths.

5. All SDS-PAGE samples are prepared by incubating protein in 50 m*M* Tris-HCL (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 m*M* dithiothreitol (DTT) for 10 min at 37°C prior to gel loading. Boiling of GF samples is avoided as this results in protein aggregation to such a degree that a large portion is retained at the interface between the stacking and resolving gel and in the sample well itself. This is a problem commonly associated with boiling of membrane proteins ***(31)***. Separation is achieved by SDS-PAGE in Laemelli discontinuous gels ***(30)*** composed of a 2.5% acrylamide stacking gel and a 10% resolving gel. After electrophoresis, proteins are visualized either by Coomassie staining or by immunodetection following electrophoretic transfer to a nitrocellulose membrane using the Mini Trans-Blot® Cell from Bio-Rad. Following the protocol described by Novagen (***14***, *see also* ***29***), the nitrocellulose is incubated in anti-T7-antibody alkaline phosphatase conjugate which binds to the 11 amino-acid T7 epitope on the amino-terminus of the recombinant GF. Immunoreactive proteins are visualized by color development in nitro blue tetrazolium (334 μg/mL) and 5-bromo-4-chloro-3-indolylphosphate (175 μg/mL) in a buffer composed of 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, and 1 m*M* $MgCl_2$.
6. The spectropolarimeter is routinely calibrated using an aqueous solution of 1.0 mg/mL (4.31 m*M*) (+)-10-camphorsulphonic acid. The concentration of the calibrant is determined using the molar extinction coefficient of 34.5 $L \cdot mol^{-1} \cdot cm^{-1}$ at 285 nm and the M_r of 232.3 ***(35)***. Wavelength calibration is done using the positive band at 290.5 nm and the negative band at 192.5 nm. Millivolt output is converted to millidegrees using the known ellipticity of camphorsulphonic acid at 290.5 nm ($\Delta\epsilon = 2.36\ L \cdot mol^{-1} \cdot cm^{-1}$; $\Delta A = 5.08 \times 10^{-4}$; $\theta = 16.76$ millidegrees) in a 0.05-cm pathlength cell. A second calibration can be done at 192.5 nm ($\Delta\epsilon = -4.9\ L \cdot mol^{-1}\ cm^{-1}$; $\Delta A = -1.05 \times 10^{-3}$; $\theta = -34.6$ millidegrees). If the ratio of the ellipticities measured at 192.5 nm and 290.5 nm is smaller than 2.0 this indicates that the instrument is not performing well at the lower wavelengths.

Acknowledgment

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9

Solubilization of Chemokine Receptors from Cell Membranes

Robert Staudinger and Juan C. Bandrés

1. Introduction

Detergent solubilization is a crucial step for the purification and biochemical, biophysical, and structural characterization of receptor molecules. The dependence of the native conformation of these proteins on the hydrophobic, intramembrane environment has complicated attempts to isolate those integral surface receptor molecules. Irreversible protein denaturation may occur during solubilization of the cell membrane. Peripheral or membrane-associated proteins have been partially or selectively extracted by various procedures such as use of chelating agents or protein perturbants, manipulations of ionic strength, or pH and enzymatic digestions ***(1)***.

The typical experimental approach for solubilizing a receptor from the cell membrane is to use biological detergents, which are agents whose physical properties resemble those of the lipophilic environment of the membrane bilayer. In the solubilization procedure, the choice of detergent is critical, because the solubilized receptor must retain at least some of its relevant properties, most importantly, its native ligand-binding properties because this is the way to "trace" a solubilized protein throughout the subsequent steps of purification ***(2)***. Although the literature on solubilization procedures is vast, the use of detergents has largely been empirical. Although we cannot review detergent properties and detergent/lipid interactions in this chapter, we will provide some suggestions on the choice of these critical components of the solubilization process. Detergents have three distinguishing characteristics: structure of the molecule, size of the micelle, and critical micellar concentration (CMC), which is the concentration above which micelles form. Both nonpolar and polar parts of detergents are highly variable. The shape of the molecule determines the pack-

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ing arrangement and thus, the size of the micelle and the interaction between the polar groups determines the CMC. Detergent molecules exist as monomers and micelles in aqueous solution. Above the CMC, most of the excess detergent will form micelles rather than monomers. This is desirable when one wants to remove detergent by dialysis or replace it with another detergent. However, in general, detergents with a large CMC and a high concentration of monomers will denature proteins more readily than those with a low CMC. On the other hand, detergents with a low CMC may not be able to solubilize the protein. The properties of detergents are reviewed in detail elsewhere ***(3,4)***.

By definition, a protein is solubilized if it remains in the supernatant after centrifugation at 100,000*g* for 1 h. For a successful solubilization process, the amount of detergent or the ratio of detergent to lipid and protein of the membrane is essential. In general, to obtain maximal exchange of lipid for detergent around the proteins, at least 10 mg of detergent per mg of lipid should be used. The detergent concentration must be sufficiently above the CMC to provide approx 1.5–2 micelles per protein molecule, otherwise proteins may be trapped in the same micelles. Although efficient in disrupting lipid–lipid and lipid–protein interactions in membranes, nonionic detergents are ineffective in breaking interactions among proteins and can thus be used to study the subunit structure of membrane proteins or their association with extrinsic proteins; however, nonspecific aggregation of proteins in the presence of detergent should be excluded ***(5)***.

Before the solubilization process, one must have a reliable assay in place to detect the activity of the solubilized protein. However, the presence of the detergent may interfere with the detection method and the assay condition must be modified ***(6)***. If the protein is a cell surface receptor, a method for the separation of bound from free ligand in solution must be developed. Precipitation of receptor with polyethylene glycol is often used for this purpose. However, if the ligand is a protein itself, this method may not be useful. Here, we describe as an example, the successful solubilization of the chemokine- and HIV-1 co-receptor CXCR4 ***(7)*** and share our experience with this procedure. We have used this same protocol for the solubilization of other chemokine receptors (IL-8) so it can be used for the solubilization of chemokine receptors (as well as other membrane receptors with transmemebrane domains) in general.

2. Materials

2.1. Crude Membrane Preparation

1. CEM cells (*see* **Note 1**).
2. Lysis buffer: 50 m*M* HEPES, pH 7.4, 1 m*M* EGTA containing protease inhibitor cocktail (Sigma).
3. Dounce homogenizer, tight pestle.

2.2. Binding Experiments with Membrane Preparations

1. Assay Buffer A: 20 m*M* HEPES, pH 7.4, 1 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 150 m*M* NaCl, and 1% BSA.
2. [^{125}I]SDF-1α (NEN Life Science Products).
3. SDF-1α (Peprotech).
4. Whatman GF/C glass fiber filters.
5. Wash Buffer A: 20 m*M* HEPES, pH 7.4, 1 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, and 500 m*M* NaCl.

2.3. Solubilization Protocol (see Note 2)

1. *n*-dodecyl-β-D-maltoside (DDM; Roche).
2. Cholesteryl-hemisuccinate (CHS; Tris-Hcl salt, Sigma).
3. Solubilization buffer: 2% DDM (w/v) and 0.25% CHS (w/v) in 20 m*M* HEPES, pH 7.4, 1 m*M* EGTA, 5 m*M* $MgCl_2$, 20% glycerol (v/v), and protease inhibitor; always prepare fresh; mix gently in an end-over-end rotator over night at 4°C. CHS is difficult to dissolve; mix 1:1 with membrane protein suspension; avoid foaming, which may denature proteins (*see* **Note 3**).
4. CHAPS (Pierce).
5. Cyclo-hexyl-pentyl-β-D-maltoside, (Zymal™ −5, Anatrace).

2.4. Binding Assay for Solubilized CXCR4

1. Assay Buffer B: 20 m*M* HEPES, pH 7.4, 1 m*M* EGTA, 5 m*M* $MgCl_2$, 1% BSA, 150 m*M* NaCl, and protease inhibitor cocktail.
2. Whatman GF/F glass fiber filters.
3. Wash Buffer B: 20 m*M* HEPES, pH 7.4, 1 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 500 m*M* NaCl, 0.1% BSA, and 0.1% DDM.

3. Methods

3.1. Crude Membrane Preparation

1. Rinse CEM cells with phosphate-buffered saline (PBS).
2. Resuspend the cells in lysis buffer and homogenize gently with 40 strokes with a tight pestle in a Dounce homogenizer. Check lysis of cells under the microscope; nuclei should be intact.
3. Spin the suspension at low speed (800*g* for 10 min at 4°C) to pellet nuclei and unbroken cells.
4. The supernatant is then centrifuged at 45,000*g* for 30 min at 4°C.
5. Wash the crude membrane pellet once and then centrifuge at 45,000*g* for 30 min at 4°C.
6. Resuspend the pellet in lysis buffer with the aid of a Dounce homogenizer, quickly freeze in methanol-dry ice, and store at −80°C.

3.2. Binding Experiments with Membrane Preparations

1. Incubate 15–20 μg of CEM crude membrane protein with 0.5–0.6 n*M* of ^{125}I-SDF-1α in Assay Buffer A, in a volume of 100 μL at 4°C for 3 h (*see* **Note 4**). It is essential to include NaCl in the assay buffer, since SDF-1α is quite "sticky." Define nonspecific binding with 500 n*M* SDF-1α.
2. Separate receptor bound radioligand from unbound ligand by rapid filtration through Whatman GF/C filters, presoaked in 0.3% polyethylenimine. Whatman GF/C filters work best for membrane-bound receptors. Rinse filters twice with 4 mL of ice-cold Wash Buffer A and then determine filter bound radioactivity in a γ-counter.
3. To determine the affinity of SDF-1α for membrane-bound CXCR4, generate full homologous competition curves. For this purpose, mix a fixed concentration of ^{125}I-SDF-1α with increasing concentrations of unlabeled SDF-1α. Not more than 10% of total radio-ligand should be receptor bound. If a ligand has very high affinity, you may increase the assay volume in order to decrease its concentration. Because ^{125}I-SDF-1α has a very high specific activity (2200Ci/mmol), only a "cold saturation" technique (mixing radiolabeled SDF-1α with increasing concentrations of unlabeled SDF-1α) can be used to determine its affinity. It is assumed that labeled and unlabeled SDF-1α have identical binding properties.
4. Vary the concentration of unlabeled ligand from 0.1 to 10 times K_D (dissociation constant).

3.3. Solubilization Protocol

1. Mix the CEM membrane preparation (at a protein concentration of 4–4.5 mg/mL) 1:1 with solubilization buffer containing protease inhibitor cocktail.
2. Incubate the mixture gently at 4°C for 30 mins in an end-over-end rotator, then subject to ultracentrifugation at 100,000*g* for 60 min.

3.4. Binding Assay for Solubilized CXCR4

1. Measure ^{125}I-SDF-1α binding to solubilized receptor in Assay Buffer B, containing 75–90 μg of solubilized protein and 0.37–0.4 n*M* ^{125}I-SDF-1α in a volume of 100 μL. Again, inclusion of NaCl in the assay buffer is essential for specific binding. The final detergent concentration should be reduced to no more than 0.3% (*see* **Note 5**). Nonspecific binding is determined by inclusion of 500 n*M* unlabeled ligand.
2. Incubate for 3 h at 4°C with the solubilized receptor and then recover bound radioligand by rapid filtration through Whatman GF/F filters, presoaked in 1% polyethylenimine for 3 h, followed by one rapid rinse with Wash Buffer B (*see* **Note 6**).
3. As for membrane-bound CXCR4, incubate a fixed concentration of ^{125}I-SDF-1α with increasing concentrations of unlabeled SDF-1α to determine the affinity by Scatchard analysis.

We tested several detergents, including CHAPS, digitonin, Cymal-5™ and DDM/CHS for their ability to extract CXCR4 with functional binding properties for SDF-1α. Only DDM/CHS, and to a lesser extent, CHAPS and Cymal™-5

were successful in releasing the binding sites in an active form. A single class of binding sites was found in Scatchard analysis and the slope of the competition curve was not different from unity (n_H=0.92 ± 0.05). We calculated a dissociation constant of 5.7 ± 0.9 n*M* and a binding capacity of 365 ± 25 fmol/mg of protein. Detergent solubilization resulted in the extraction of 56–62% protein and 11–15% of active binding sites. The solubilized receptor remained stable at 4°C without loss of binding activity for at least 1 wk.

4. Notes

1. Successful solubilization of cell surface receptors requires a rich source of the protein. In our case the cell line CEM expressed relatively high levels of the HIV-1 coreceptor CXCR4 (2.69 pmol/mg of membrane protein) ***(7)***. In case of the second principal HIV-1 coreceptor CCR5 a codon-optimized canine thymocyte cell line was generated, expressing high levels of CCR5 ***(8)***. If the tissue of interest expresses too low levels of receptor and an immortalized cell line instead is used to purify the relevant receptor, it is important to ensure that the binding sites are identical by comparing the physicochemical and pharmacological properties of the receptors ***(9)***.
2. Commercial detergents are as a rule chemically impure and heterogeneous. Even one batch may differ from the next. It is advisable to purchase high quality detergents. In our experience, detergents from Boehringer Mannheim, Pierce, and Anatrace are of excellent quality. If a particular detergent works for the solubilization of your receptor, reorder the same batch.
3. Inclusion of glycerol, EGTA or ammonium sulfate may increase the stability of the solubilized receptor.
4. Although it is sometimes advisable to determine efficiency of solubilization at different temperatures, it is our experience that integral membrane proteins, in particular G-protein coupled receptors (GPCR) with several transmembrane domains, do not tolerate temperatures above 4°C, at which irreversible denaturation may occur. Therefore, it may be necessary to carry out all steps of the solubilization and purification process at 4°C or in ice. If an agonist to a GPCR is used to follow the activity of the receptor during solubilization, one needs to consider that binding of an agonist to a GPCR is highly temperature dependent ***(10,11)***. Reduction of incubation temperature from 20°C to 4°C resulted in a 58% inhibition of SDF-1α binding to CXCR4, which was due to a conversion of high affinity binding sites to a lower affinity state ***(11)***. However, the lower affinity SDF-1α binding site is still detectable. In contrast, reduction of the incubation temperature from 20°C to 4°C resulted in inhibition of MIP-1β binding to CCR5, the second principal HIV-1 coreceptor, by 95% ***(12)***. Presumably, the affinity of the low affinity state of CCR5 for MIP-1β was beyond the limits of detection in our binding assay and we were unable to demonstrate activity of CCR5 after solubilization (unpublished observation).
5. The detergent may interfere with detection of the solubilized receptor. It is usually necessary to further reduce the final detergent concentration, however, aggregation

or precipitation of the solubilized protein may occur below the CMC of the detergent. Although we mentioned earlier that the usefulness of a detergent is often determined empirically and it may be advisable to compare detergents from each chemical category, digitonin, CHAPS, n-dodecylmaltoside plus cholesteryl-hemisuccinate, and Zymal-5™ were successfully used for solubilization of receptors of the G-protein coupled superfamily. When performing exploratory experiments with various detergents and solubilization conditions, it is important to account for the recovery of all biological activity. Determine whether a detergent (at different incubation periods and possibly temperatures) is able to release a prelabeled receptor into the supernatant after centrifugation at 100,000*g*. Determine if the radioactivity is free or receptor-bound and keep an exact balance of the receptor activity. In control experiments, dissociation of the labeled ligand from its receptor during the course of the solubilization procedure should be assessed. In this way, the effectiveness of a detergent in solubilizing a particular receptor or the inactivation of the receptor can be effectively determined.

6. As aforementioned, a method to detect receptor activity must be developed to follow the solubilized protein after the solubilization procedure. The easiest and most convenient procedure is the rapid filtration technique. Usually, receptor-bound ligand can be precipitated with polyethylene glycol (PEG) and γ-globulin as a carrier protein ***(13)***. The solubilized receptor-ligand complex is retained on glass fiber filters. However, coprecipitation of free ligand can be a serious problem with the PEG precipitation method. The proper concentration of PEG required to precipitate the receptor-ligand complex should be determined for each radioligand under your own conditions. When we tested the PEG precipitation method in a binding assay for solubilized CXCR4, variations in PEG concentration or ionic strength of the filtration method did not result in the preferential precipitation of the receptor-ligand complex. Therefore, if the ligand of a receptor is a peptide, this technique may not be helpful. A modification of the routine filtration method was however useful in the binding assay for solubilized CXCR4. Glass fiber filters (Whatman GF/F) presoaked in 0.3% polyethylenimine for 2 h retained the CXCR4-^{125}I-SDF-1α complex with little retention of free ^{125}I-SDF-1α. The pretreated filters should be placed into the filtration machine without rinsing.

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10

A Systematic Approach for the Solubilization of the Integral Membrane Protein Lysophospholipid

Acyl-Coa Acyltransferase (LAT)

Claus Kerkhoff and Volkhard Kaever

1. Introduction

The lysophospholipid:acyl-CoA acyltransferase (LAT, EC 2.3.1.23) catalyzes the reacylation of lysophospholipids with coenzyme A-activated fatty acids, and is part of the Lands cycle ***(1)***. The LAT is an important enzyme participating in the rapid turnover of phospholipids thereby: 1) maintaining the membrane lipid composition and the asymmetrical distribution of unsaturated fatty acids within the phospholipids (for review, *see* **refs. *2,3***), and 2) controlling the free arachidonic acid levels (for review, *see* **ref. *4***). In addition, LAT is thought to play a crucial role in the early phase of T-cell activation by the elevated incorporation of polyunsaturated fatty acids into plasma membrane phospholipids ***(5,6)***. LAT is suggested to be an integral membrane protein, and it is established that there are separate acyltransferases with different specificity toward both lysophospholipids and acyl-CoAs ***(7,8)***.

Despite a great deal of effort by several groups, little progress has been made so far in the purification of LAT because of the enzyme's instability and its sensitivity toward detergents. The solubilization of functional membrane proteins is the critical first step in protein purification (for review, *see* **refs. *9,10***) because the substitution of lipids by detergent may cause enzyme inactivation. Moreover, membrane proteins are extremely sensitive to their hydrophobic environment. Thus, there is no single detergent or solubilization scheme that is equally applicable to all membrane proteins.

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However, two general considerations have to be explored in a systematic approach for the solubilization of a membrane protein ***(11)***: First, initial solubilization experiments have to survey conditions that extract membrane proteins from membranes as protein/lipid/detergent mixed micelles and maintain the enzyme activity. Once solubilization has been achieved, the further effects of changes in detergent concentration on size and composition of the solubilized complexes should be examined to yield protein/detergent complexes that are free of lipid and accessible to protein purification.

Here, we describe a four-step protocol by which experimental conditions were defined that allowed: i) the extraction of the integral membrane protein LAT from membranes; ii) its reconstitution with artificial membranes, thereby restoring the LAT enzyme activity; iii) the development of a solubilization protocol by which protein solubilization was achieved under the preservation of the full enzyme activity; and iv) the dissociation of the protein/lipid/detergent mixed micelles, thereby yielding protein/detergent complexes with partial enzyme activity. These mixed micelles were accessible to protein purification, and we are currently analyzing the protein composition of a LAT candidate protein.

2. Materials

2.1. LAT Standard Enzyme Assay

1. LAT assay buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* ethylenediamine tetraacetic acid (EDTA).
2. 1-Palmitoyl-GPC was from Fluka-Biochemika (Neu-Ulm, Germany). Coenzyme A was from Boehringer (Mannheim, Germany). Arachidonoyl chloride was from Nu Chek Prep (Elysian, Minnesota, USA). [^{14}C]-1-palmitoyl-GPC (spec. act. 55 mCi/mmol) was from NEN/DuPont (Bad Homburg, Germany). Fatty acid-free bovine serum albumin was from Sigma (Taufkirchen, Germany). 2,6-di-t-butyl-4-methylphenol was from Sigma (Taufkirchen, Germany). Scintillation cocktail (Emulsifier-Safe) was from Canberra Packard (Frankfurt/Main, Germany).
3. Silica gel plates (Merck HPTLC 60) were from Merck.
4. Phospholipid solvent system: chloroform : methanol : deionized water : acetic acid (50:25:2.5:8, v/v/v/v).
5. Coomassie blue stain solution: 100 m*M* NaCl, 25% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue G-250

2.2. Extraction of LAT from Crude Membranes

1. Homogenization buffer: 250 m*M* sucrose, 50 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 20% (w/v) ethylene glycol.
2. LAT assay buffer (*see* **Subheading 2.1.**).

2.3. Reconstitution of LAT Enzyme Activity with Artificial Membranes

1. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were from Sigma (Taufkirchen, Germany).
2. LAT assay buffer (*see* **Subheading 2.1.**).

2.4. Development of an Optimal Solubilization Protocol

1. LAT assay buffer (*see* **Subheading 2.1.**).
2. CHAPS, OGP (n-octyl glucopyranoside), Triton X-100, sodium cholate, and sodium deoxycholate were from Sigma (Taufkirchen, Germany).

2.5. Dissociation of the Protein/Lipid/Detergent Mixed Micelles

1. 30% (w/v) acrylamide-*bis*-acrylamide mixture (T = 30%, C = 0.8% Roth, Karlsruhe, Germany).
2. 1.5 *M* Tris-HCl, pH 8.8.
3. 40% (w/v) ammonium persulfate solution.
4. TEMED.
5. Electrophoresis buffer: 50 m*M* Tris-HCl, pH 8.8.

3. Methods

3.1. LAT Standard Enzyme Assay

The lysophosphatidylcholine:acyl-CoA acyltransferase activity is measured by the formation of [^{14}C]-PC using arachidonoyl-CoA and [^{14}C]-lyso-PC as substrates. Arachidonoyl-CoA is available from commercial sources. Alternatively, arachidonoyl-CoA is synthesized according to Reitz et al. ***(12)***, using arachidonoyl chloride and Coenzyme A. Immediately after termination of the reaction 1 mole 2,6-di-t-butyl-4-methylphenol to 2 mol arachidonoyl-CoA is added as antioxidant. The arachidonoyl-CoA is dissolved in LAT assay buffer, and aliquots are frozen at −80°C (*see* **Note 1**).

1. The complete assay mixture contains 0–50 μg of protein, 50 μmol/L [^{14}C]-lyso-PC (5,000 dpm/nmol), 30 μmol/L arachidonoyl-CoA, and 12.5 μmol/L fatty acid-free bovine serum albumin (BSA), and LAT assay buffer to a final volume of 200 μL.
2. The reaction mixture is preincubated for 15 min at 4°C, and the reaction is initiated by incubation at 37°C.
3. After 30 min, the reaction is stopped by the addition of 1.5 mL methanol : chloroform (2:1, v/v) and 200 μL deionized water.
4. Lipids are extracted according to the method of Bligh and Dyer ***(13)***. Briefly, after 1 h incubation at room temperature, the phase separation is caused by the addition of 0.5 mL deionized water and 0.5 mL chloroform followed by brief centrifugation for 10 min at 400*g*. The upper organic phase is removed and dried under vacuum.

5. Nonlabeled PC (20 μg in 20 μL chloroform:methanol = 2:1v/v) is added as standard and the extracts are carefully vortexed. Aliquots (5 μL) are applied onto 20 × 20 cm thin-layer chromatographic silica gel plates, and the plates are developed with chloroform : methanol : deionized water : acetic acid (50:25:2.5:8, v/v/v/v) solvent system.
6. The lipids are visualized according to the method of Nakamura and Handa ***(14)***. Briefly, the plates are air-dried and then are dived in a tank containing a solution of 100 m*M* NaCl, 25% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue G-250.
7. The areas corresponding to PC and lyso-PC are scraped from the plates and the radioactivity is measured after transfer to scintillation vials containing 10 mL of scintillation cocktail.

Enzyme activity is given as nmol [^{14}C]-PC formed per 30 min. All assays are performed in duplicate or triplicate. The rate of incorporation of arachidonoyl-CoA into lyso-PC is linear for at least 15 min, has a broad pH range from 6.0 to 8.8, and is not affected by calcium concentrations up to 5 m*M* (data not shown).

3.2. Extraction of LAT from Crude Membranes

In the initial solubilization experiment, crude membranes, prepared from pig spleen by differential centrifugation similar as described by Szamel et al. ***(15)***, are treated with various detergents in the presence and absence of 0.5 mol/L NaCl (*see* **Note 2**). The remaining LAT enzyme activity is then measured in the membranes. (Note: LAT enzyme activity of the solubilized fractions is measured after reconstitution with artificial membranes as described in **Subheading 3.3.**)

1. Pig spleen (400 g) is washed in ice-cold homogenization buffer, minced, and homogenized in 400 mL homogenization buffer using a loose-fitting motor-driven glass/Teflon homogenizer.
2. The homogenate is first filtered through cotton mesh and then centrifuged at 15,000*g* for 60 min at 4°C.
3. The supernatant is centrifuged again at 200,000*g* for 90 min at 4°C. The pellet is washed with homogenization buffer, resuspended in LAT assay buffer to a protein concentration of 50 mg/mL, and stored frozen in aliquots at −80°C. Upon storage for 4 wk, nearly 90% of the original LAT activity was recovered.
4. For solubilization of LAT the crude membranes are resuspended in LAT assay buffer to a final protein concentration of 10 mg/mL and treated with combinations of increasing concentrations of detergent and 0.5 mol/L NaCl as indicated.
5. The mixtures are then incubated at 4°C for 60 min with constant stirring, and centrifuged at 100,000*g* for 60 min at 4°C. Aliquots are applied to the LAT standard enzyme assay as described in **Subheading 3.1.**

The solubilization is completed within this time frame. The enzyme activity located in the supernatant immediately after centrifugation is regarded as "solubilized."

3.3. Reconstitution of LAT Enzyme Activity with Artificial Membranes

Solubilized membrane proteins have been shown to retain full enzyme activity after reconstitution with artificial membranes ***(16–18)*** (*see* **Note 3**).

1. 5 mg PC, 3 mg PE, and 1 mg PS are dissolved in chloroform.
2. The solution is dried under N_2. The lipids are then reconstituted in 1 mL LAT assay buffer and sonicated at 4°C for 5 min at 20 W to obtain liposomes.
3. After centrifugation at 1000*g* for 10 min at 4°C the liposomes are mixed with the solubilized proteins at ratios from 1:1 to 1:10 (w/w).
4. To remove the detergent, the solution is subsequently dialyzed against LAT assay buffer overnight at 4°C followed by LAT standard enzyme assay.

3.4. Development of an Optimal Solubilization Protocol

For the development of an optimal solubilization protocol, we treat P2 membranes with increasing detergent concentration at different salt concentrations. P2 membranes contain the integral LAT (iLAT) that differs from the peripheral LAT in its topological localization and its specificity toward CoA-activated fatty acids ***(19)***. Whether both enzyme activities represent different acyltransferases still remains unclear (*see* **Note 4**).

1. Crude membrane suspensions containing 10 mg/mL protein each are treated with solutions of high ionic strength (LAT assay buffer supplemented with 1 *M* NaCl) for 60 min at 4°C. The solubilization is completed within this time frame.
2. The mixtures are centrifuged at 100,000*g* for 90 min at 4°C to obtain the supernatant (S1) and the pellet (P1).
3. The pellets P1 are resuspended in LAT assay buffer containing 10 m*M* CHAPS to a final protein concentration of 10 mg/mL and again centrifuged at 100,000*g* for 60 min at 4°C.
4. The resulting pellets P2 are resuspended in LAT assay buffer to a final protein concentration of 10 mg/mL and treated with combinations of increasing concentrations of detergent and NaCl as indicated in **Fig. 1**.
5. The mixtures are incubated at 4°C for 60 min with constant stirring, and centrifuged at 100,000*g* for 60 min at 4°C. The solubilization is completed within this time frame.

The enzyme activity located in the supernatant immediately after centrifugation is regarded as "solubilized." The LAT enzyme activity measurements are performed with aliquots of the different supernatants as described later. The

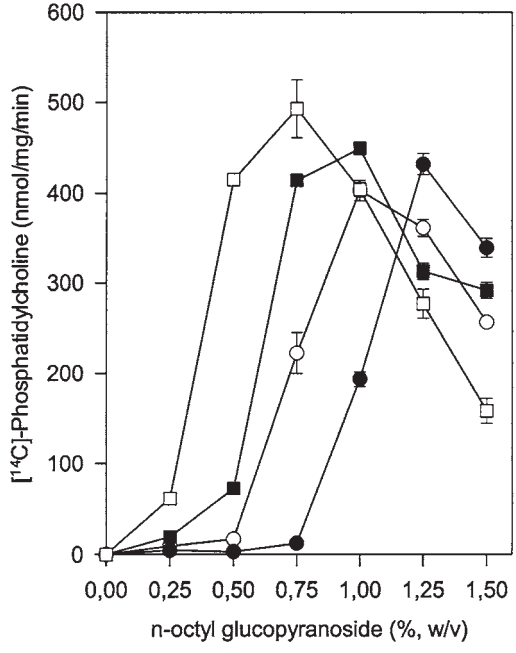

Fig. 1. Solubilization of P2 membranes with different concentrations of OGP and NaCl. P2 membranes from human placenta were prepared as described in experimental procedures, suspended in LAT buffer to a final protein concentration of 10 mg/mL and treated with combinations of increasing NaCl and OGP concentrations for 60 min at 4°C as indicated. After centrifugation LAT enzyme activity in the resulting supernatants was assayed by the formation of [^{14}C]-PC with 50 μ*M* [^{14}C]-lyso-PC (5000 dpm/nmol) and 30 μ*M* arachidonoyl-CoA. Lipids were extracted and separated by TLC. The LAT enzyme activity is expressed as nmol formed [^{14}C]-PC per min and mg protein. The bars represent mean data ± range from duplicate determinations of a representative experiment from three similar ones. (-●-) 150 m*M* NaCl; (-○-) 650 m*M* NaCl; (-■-) 1,150 m*M* NaCl; (-□-) 2,150 m*M* NaCl.

(The figure is taken from **ref. *31*** with permission of Blackwell Science.)

supernatant S1 contains a distinct LAT enzyme activity that we refer to as peripheral LAT (pLAT) ***(19)***.

3.5. Dissociation of the Protein/Lipid/Detergent Mixed Micelles

To dissect the resulting protein/lipid/detergent mixed micelles, different amounts of CHAPS are added to aliquots of the dialyzed protein-lipid-OGP mixed micelles to yield detergent-to-protein ratios from 1:1 to 20:1. Aliquots

are analyzed upon their LAT enzyme activities (*see* **Note 5**). The molecular sizes of the protein/detergent micelles are analyzed by native polyacrylamide gel electrophoresis (PAGE). For native PAGE, a uniform polyacrylamide concentration (5%) is used in conjunction with a single homogeneous buffer system according to Andrews ***(20)*** with some modifications.

1. Briefly, 2.5 mL of 30% (w/v) acrylamide-*bis*-acrylamide mixture, 3 g glycerol, 0.5 mL of 1.5 *M* Tris-HCl, pH 8.8, and 9 mL deionized water are mixed and polymerized overnight by addition of 15 μL TEMED and 15 μL of a 40% ammonium persulfate solution.
2. Bromphenol blue is added to the protein sample (40 μg/10 μL) as tracking dye.
3. The electrophoresis is started at 70 V. After about 1 h, when the sample has completely entered the gel, the running conditions are set to 200 V.
4. For LAT enzyme activity measurements, the gel is cut into pieces, and the gel pieces are each incubated with 200 μL LAT assay buffer containing 10 nmol [^{14}C]-lyso-PC, 6 nmol arachidonoyl-CoA, and 1.5 nmol fatty acid BSA for 30 min at 37°C.
5. The lipids are extracted and analyzed as described for the LAT standard enzyme assay.

4. Notes

1. The assay with [^{14}C]-lyso-PC and unlabeled acyl-CoA is not recommended when there is a substantial amount of lyso-PC:lyso-PC acyltransferase activity in the enzyme preparation. The spectrophotometric assay is more rapid, but less specific due to the principle of the assay to determine free thiol groups ***(7)***.

   In addition to the complexity to define experimental conditions for an integral membrane protein allowing solubilization under the preservation of the enzyme activity, there are further requirements to take into consideration. The lysophospholipid:acyl-CoA acyltransferase (LAT) catalyzes the reaction of two hydrophobic molecules. The detergent-like effects of both long chain acyl-CoAs and 1-acyl-GPL must be considered when utilizing an in vitro-assay system. Ratios of 1:4 (mol/mol) for albumin and arachidonoyl-CoA were found to be most effective to maintain the LAT enzyme activity and to minimize the detergent-like properties of long chain acyl-CoAs ***(19)***. In addition, 1-acyl-GPLs represent amphiphilic molecules that form monomers only at low concentrations. At concentrations above their critical micellar concentration (CMC), they form micelles. It has been suggested that a micellar substrate is not utilized by acyltransferases ***(21)***. The addition of organic solvents or detergents to increase their water solubility is disadvantageous because it has been shown in several reports that LAT activity is sensitive to both detergents and organic solvents. To overcome this fact, we have earlier reported the use of n-octyl glucopyranoside (OGP) as a dispersing agent for the hydrophobic and amphiphilic lipid substrates in the LAT enzyme activity assay ***(8)***. At low concentrations below its CMC (final concentration of 0.1% [w/v]) OGP exhibited no inhibitory effect upon LAT enzyme activity.

2. Choice of the optimum type and concentration of detergent for the successful solubilization of most integral membrane proteins is still very much a matter of trial and error. As documented in the literature various detergents have been tested for their ability to solubilize LAT from membranes such as lyso-PC ***(22,23)***, Triton X-100 ***(24,25)***, sodium deoxycholate ***(26–28)***, and CHAPS ***(29)***. However, despite a great deal of effort by several groups, little progress has been made so far in the purification of LAT.

   One reason might be that detergents at higher concentrations do not improve the solubilization and cause an inactivation of enzyme activity. In order to reduce any inactivating effect of detergents upon enzyme activity, we performed initial studies with various detergents in the presence and absence of 0.5 *M* NaCl ***(19)***. High ionic strength reduces the CMC significantly and the combination of detergent and high ionic strength may enhance the solubilizing effect of the detergent. Consequently, the detergent concentration can be reduced and the inactivating effect of detergents upon enzyme activity may be minimized.

   In our initial studies using membranes from pig spleen we compared the activities of the detergent-insoluble enzyme in the resulting membrane pellet at the same detergent concentration in the presence and absence of 0.5 *M* NaCl (*see* **Table 1**). Triton X-100 had a strong inactivating effect on enzyme activity at low concentrations, lyso-PC was ineffective to solubilize LAT from the membranes up to about 500 μmol/L. Sodium deoxycholate had a remarkable inactivating effect on enzyme activity and was little effective to solubilize LAT from the membranes. Sodium cholate had a slight inactivating effect on enzyme activity but was little effective to solubilize LAT from the membranes, too. In these experiments n-octyl glucopyranoside (17 mM) had a very little inactivating effect on LAT activity and was found to be most effective for the solubilization of the enzyme from the membranes. Up to 83% of the total activity was solubilized when remaining LAT enzyme activities in the membrane pellet after detergent treatment in the absence and presence of NaCl were compared.
3. Enzyme activity of integral membrane proteins has been successfully restored by reconstitution with artificial membranes ***(16–18)***. Therefore, the various detergent-solubilized membrane proteins were reconstituted with artificial membranes (liposomes) to exclude the possibility that the different supernatants contained inactive enzymes because of the detergent treatment. We prepared liposomes (PC:PE:PS=5:3:1, w/w/w) under consideration of the asymmetrical distribution of phospholipids in membranes ***(30)***.

   The detergent-solubilized membrane proteins were mixed with liposomes at the ratio 1:10 (protein : lipid, w:w) for reconstitution, and the LAT standard assay was performed with aliquots (*see* **Table 1**). After reconstitution the sodium deoxycholate supernatants contained no LAT enzyme activity, and the sodium cholate supernatant contained only little LAT enzyme activity. Up to 25% of the total activity was found in the n-octyl glucopyranoside supernatant, but the specific activity of the enzyme was reduced to one-third of the original activity. Higher concentrations of n-octyl glucopyranoside did not improve the solubilization and after reconstitution LAT enzyme activity was undetectable ***(19)***.

Table 1
Solubilization of LAT from Pig Spleen Crude Membranes

	%(w/v)	conc. m*M*	LAT activity in the pellet after detergent treatment [nmol min^{-1} mg^{-1}]		LAT activity in the super natant after reconstitution [nmol min^{-1} mg^{-1}]
	%(w/v)	conc. m*M*	LAT buffer	LAT buffer + 0.5 *M* NaCl	
Control			13.037		
Triton X-100	0.5		0.314	0.249	n.d.
Lyso-PC		0.5	10.228	9.636	n.d.
Sodium cholate	1.5	34.8	11.372	6.293	0.01 ± 0.01
Sodium deoxycholate	1.5	36.3	4.492	2.501	0.04 ± 0.01
Sodium deoxycholate	3.0	72.6	3.467	1.760	0.14 ± 0.02
Octyl glucopyranoside	0.5	17.0	11.354	0.575	0.96 ± 0.03

Crude membranes from pig spleen were diluted in LAT buffer in the presence and absence of 0.5 mol/L NaCl to a final protein concentration of 10 mg/mL and treated with various detergents as indicated. After incubation for 30 min at 4°C and centrifugation at 100,000*g* for 60 min at 4°C the pellets were resuspended in LAT buffer, and the supernatants were mixed with liposomes (PC:PE:PS=5:3:1; w/w/w) at a ratio of 1:10 (protein/lipid, w/w). In both pellets and supernatants the LAT enzyme assay was measured. The LAT enzyme activity is expressed as nmol formed [^{14}C]-PC per min and mg protein.

Table 2
Reconstitution of Solubilized LAT with Liposomes at Different Molar Ratios

Molar ration lipid:protein % (w/v)	LAT enzyme activity ol min^{-1} mg^{-1}]
Control	2.88 ± 0.03
1:10	5.62 ± 0.06
1:5	6.71 ± 0.24
1:2.5	8.58 ± 0.93
1:1	10.00 ± 0.82

Crude membranes were resuspended into LAT buffer containing 0.5 mol/L NaCl to a final protein concentration of 10 mg/mL and treated with 0.5% (w/v) n-octyl glucopyranoside for 30 min at 4°C. After centrifugation at 100,000*g* for 60 min at 4°C the supernatant was mixed with liposomes (PC:PE:PS=5:3:1, w/w/w) at a ratio from 1:1 to 1:10 (protein/lipid, w/w) and a standard LAT enzyme assay was performed with aliquots of the different reconstituted LATs. Control is defined as the original LAT enzyme activity of the n-octyl glucopyranoside supernatant without reconstitution. The LAT enzyme activity is expressed as nmol formed [^{14}C]-PC per min and mg protein.

For further consideration of reconstitution conditions the supernatant after treatment with n-octyl glucopyranoside was mixed at ratios from 1:1 to 1:10 (protein : lipid, w/w) with liposomes (*see* **Table 2**). At 1:10 ratio, the LAT enzyme activity was slightly enhanced compared to the LAT enzyme activity in the supernatant. When liposomes were added to the n-octyl glucopyranoside supernatant at the 1:1 ratio, the total activity of the membranes was restored to the original LAT activity before detergent treatment. The n-octyl glucopyranoside-solubilized and reconstituted proteins were stored at 4°C. Upon storage for 4 wk, 70–90% of the original activity was recovered.

4. We used the conditions of the initial solubilization approach as basis for refinement. **Fig. 1** shows the solubilized LAT enzyme activity as a function of increasing detergent concentration at different salt concentrations. The maximum of enzyme activity depended on the ionic strength of the solubilization buffer. The maximal enzyme activity was observed at 1.25% (w/v) = 42.5 m*M* OGP in buffer containing 150 m*M* NaCl, at 1.0% (w/v) = 34 m*M* OGP in buffer containing 650 m*M* NaCl, at 0.75% (w/v) = 25.5 m*M* OGP in buffer containing 1150 m*M* NaCl, and at 0.5% (w/v) = 17 m*M* OGP in buffer containing 2150 m*M* NaCl, respectively ***(31)***. The peak of solubilized LAT enzyme activity was presumably the result of a progressive solubilization and inactivation at higher detergent concentrations. It is worthwhile to note that the amount of extracted protein did not correlate with the salt concentration at a fixed detergent concentration. The protein content in the supernatant was enhanced when the salt concentration was increased to 650 m*M* NaCl. A further increase in the salt concentration did not raise the amount of extracted protein.

The effect of increasing salt concentrations on the solubilization efficiency of OGP might be a result of the dependence of the CMC on salt concentration. It has

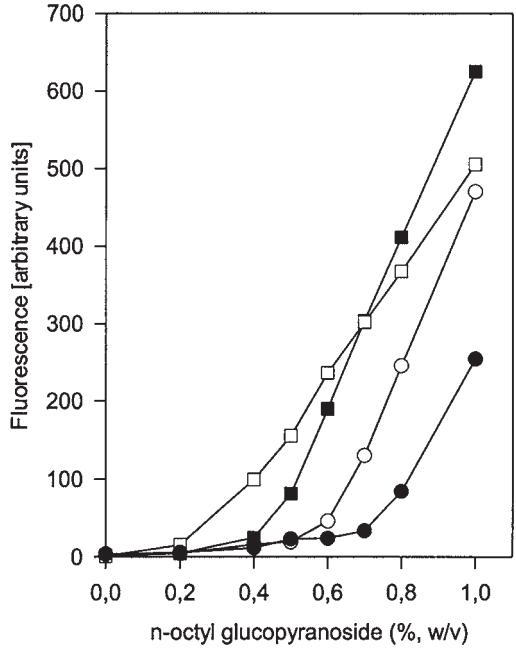

Fig. 2. DPH fluorescence as a function of concentration of CHAPS for (-●-) 0 m*M* NaCl; (-○-) 500 m*M* NaCl; (-■-) 1,000 m*M* NaCl; (-□-) 2,000 m*M* NaCl. The fluorescence scale is in arbitrary units. (The figure is taken from **ref.** ***31*** with permission of Blackwell Science.)

been shown that addition of salt drastically decreased the CMC of charged detergents such as SDS ***(32–34)*** because the ionic interactions are reduced with increasing salt concentrations leading to a more effective dissociation of the membrane protein from the nonsoluble membrane constituents as well as from other soluble molecules. Chattopadhyay and Harikumar ***(35)*** have shown that the CMC of the zwitterionic detergent CHAPS depended on the NaCl concentration. The effect of salt on the CMC of nonionic detergents has been expected to be less pronounced because of the absence of charge interactions ***(36)***. However, the determination of the CMC at different salt concentrations using a method developed by Chattopadhyay and London ***(32)*** revealed evidence that the CMC of the nonionic detergent OGP also depended on the salt concentration. **Fig. 2** shows the dependence of DPH fluorescence upon detergent concentration for OGP at four different salt concentrations. Fluorescence was weak at low OGP concentrations, followed by a rapid rise in DPH fluorescence that took place at and above the CMC of OGP. The CMC was obtained from the intersection of the straight line through the fluorescence at low detergent concentrations with the straight line through the fluorescence values in the region of rapid intensity increase. The CMC of OGP was calculated to 24.5 m*M*

in the absence of salt ***(31)***. This is in agreement with the literature value of 23.2 m*M* as the CMC of OGP ***(37)***. When the salt concentration was increased to 500 m*M* and 1000 m*M,* the CMC progressively decreased to 21.1 m*M* and 14.0 m*M,* respectively. At 2000 m*M* NaCl concentration, a peculiar behavior of the fluorescence with increasing salt concentrations was observed that is assumed to be caused by insolubility of THF-diluted DHP at this salt concentration. In addition to ionic strength, the CMC of OGP was also sensitive to temperature: whereas 24–26 m*M* OGP (close to CMC) was sufficed to solubilize rhodopsin from membranes at 23°C, a considerably higher concentration (32–34 m*M*) was necessary to attain the same level of solubilization at 4°C ***(38)***. Furthermore, the higher the concentration of membranes, the higher the detergent concentration required for solubilization, irrespective of whether one is close to or well above the CMC.

In analogous studies using human placenta, we determined that optimal solubilization of LAT enzyme activity is achieved at 12.2 m*M* CHAPS in the presence of 2150 m*M* NaCl ***(31)***. This concentration is far in excess of the CMC of CHAPS because the CMC of the detergent was estimated to 4.1 m*M* in the presence of 1500 m*M* NaCl ***(35)***. It is obvious that the solubilization efficiency of CHAPS did not correspond with premicellar concentrations. However, in our solubilization approach, the solubilization efficiency of CHAPS depended on the ionic strength of the solubilization buffer.

It is worthwhile mentioning that the different LAT solubilization efficiencies of the various detergents may be caused by the lipids replaced by the detergent. We found that the protein-lipid-OGP mixed micelles were relatively enriched in sphingomyelin (SPM) compared to protein-lipid-CHAPS mixed micelles ***(31)***. There was no difference in their content of other phospholipids. It is obvious that the higher the salt concentration the higher the amount of extracted SPM (*see* **Table 3**). Thus, the basis for the more efficient extraction of LAT enzyme activity by OGP may be a result of the ability to extract a higher amount of SPM from membranes. Therefore, SPM may represent an essential cofactor for both the solubility and the stability of membrane proteins in aqueous media. This is in accordance with the finding that SPM-induced activation of LAT in membranes of *ras*-transformed NIH 3T3 fibroblasts ***(39)***.

5. When adding detergents with increasing concentrations to biological membranes, three stages are defined:
    i) At low ratios of detergent to membrane lipids, detergent monomers incorporate into the lipid bilayer without disruption.
    ii) If the detergent concentration is further increased and a saturation point is reached, the membrane is fragmented, and protein-lipid-detergent mixed micelles are released into the aqueous medium.
    iii) A further increase in the detergent concentration leads to a progressive delipidation of the complexes, forcing the lipid to distribute among the increasing amounts of detergent micelles.

Size-exclusion chromatography revealed evidence that the extracted protein-lipid-OGP mixed micelles from the optimized solubilization protocol represented

Table 3
Lipid Analysis of Protein-Lipid-Detergent Mixed Micelles

	0.75% (w/v) CHAPS		0.75% (w/v) OGP	
	SPM	PC	SPM	PC
150 m*M* NaCl	31 ± 03	244 ± 26	135 ± 15	185 ± 07
650 m*M* NaCl	29 ± 06	211 ± 79	186 ± 48	200 ± 57
1150 m*M* NaCl	119 ± 11	224 ± 44	183 ± 38	181 ± 48
2150 m*M* NaCl	151 ± 56	282 ± 76	316 ± 86	288 ± 33

Crude membranes from human placenta were suspended in LAT buffer containing either 0.75% CHAPS or 0.75% OGP in the presence of increasing NaCl concentrations as indicated and incubated for 60 min at 4°C prior to centrifugation. Then aliquots of the supernatants each containing 200 µg protein were delipidated, and the extracted lipids were separated on high-performance thin-layer chromatography. The contents of cupric sulfate-charred lipid bands were quantified by densiometric scanning in comparison with standards. The table represents mean data ± range from two independent experiments.

large particles in size with molecular weights on the order 0.5–1 million Da ***(31)***. Therefore, the further effects of change in detergent concentration on both size and composition of the solubilized complexes were examined. Various detergents were added to the OGP/NaCl-extracted LAT enzyme. The LAT enzyme activity measurements revealed that enzyme activity was not affected by the addition of either CHAPS or digitonin compared to control. In contrast, OGP at higher concentrations, NP40, or sodium deoxycholate had inactivating effects on the enzymes activity.

Then different amounts of CHAPS were added to aliquots of the dialyzed protein-lipid-OGP mixed micelles to yield detergent-to-protein ratios from 1:1 to 20:1. As shown in **Fig. 3**, the LAT enzyme activity decreases with increasing detergent concentrations. When the detergent-to-protein ratio was adjusted to 20:1, 10% of the original LAT enzyme activity could be determined. It is established that:

i) The further increase of the detergent results into further delipidation of the mixed micelles; and
ii) At detergent-to-protein ratios from 10:1 to 20:1 protein-detergent complexes are formed that are free of lipid ***(11)***. The observed LAT enzyme inactivation may be owing to conformational changes within the macromolecule when the lipids in the microenvironment of the enzyme are substituted by detergent.

When these CHAPS-treated mixed micelles were separated by native PAGE, two additional high-molecular-weight proteins migrated into the gel, generated by detergent treatment. It is concluded that the LAT-lipid-OGP mixed micelles were dissected by the excess of detergent. Unfortunately, the two additional Coomassie blue-stained protein bands did not express any LAT enzyme activity. Evidence that one of these two additional high-molecular-weight protein complexes may contain the LAT enzyme was derived from photoaffinity studies with [^{125}I]-18-(4'-Azido-2'-hydroxybenzoylamino)-oleoyl-CoA. The fatty acid analogue represents a competi-

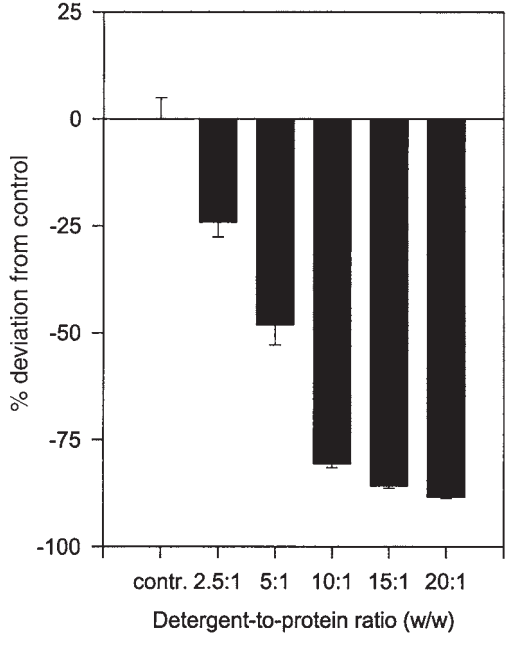

Fig. 3. LAT enzyme activity versus increasing concentrations of CHAPS. To aliquots of dialyzed protein-lipid-OGP mixed micelles (100 μg/mL) increasing amounts of CHAPS were added to yield detergent-to-protein ratios from 1:1 to 20:1 as indicated, and incubated for 30 min at 4°C followed by LAT enzyme activity measurements. The decrease of LAT enzyme activity is expressed as % deviation from LAT enzyme activity in the absence of CHAPS. The LAT enzyme activity was estimated to 552 ± 27 nmol min^{-1} mg^{-1}. The bars represent mean data from three independent experiments with duplicate determinations ± SD. (The figure is taken from **ref. *31*** with permission of Blackwell Science.)

tive inhibitor of LAT enzyme activity in the dark and an irreversible inhibitor after photolysis ***(40)***. The [^{125}I]-labeled LAT complex did not enter the gel in the absence of excess CHAPS. However, when the detergent-to-protein ratio was raised to 20:1 (w/w) a high-molecular-weight protein complex labeled by [^{125}I]-18-(4'-Azido-2'-hydroxybenzoylamino)-oleoyl-CoA co-migrated with one of the two additional high molecular weight protein complexes.

5. Conclusions and Perspectives

Despite a great deal of effort by several groups, little progress has been made so far in the LAT purification because of the enzyme instability. Here, we defined experimental conditions that allowed the extraction of LAT from membranes while maintaining the full enzyme activity. The further increase in the detergent

concentration led to a progressive delipidation of the complexes, forcing the lipid to distribute among the increasing amounts of detergent micelles, but also resulted into the partial enzyme inactivation. However, the enzyme might be accessible to further protein purification. Photolabeling with [^{125}I]-18-(4'-Azido-2'-hydroxybenzoylamino)-oleoyl-CoA may allow the purification of the [^{125}I]-labeled LAT complex. We are currently analyzing its protein composition.

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11

Purification of Omp50, a Minor Porin of *Campylobacter jejuni*

Jean Michel Bolla

1. Introduction

The porins are proteins involved in the exchange of hydrophilic solutes between Gram-negative bacteria and their outer environment ***(1).*** These proteins, which are exclusively localized in the outer membrane, are transmembranous and interact with the constituents of the membrane, lipids, and lipopolysaccharide. They also interact with the periplasmic peptidoglycan layer ***(2).***

When purified, these proteins can be reconstituted into artificial lipid bilayers (proteoliposomes or black lipid bilayers) in order to analyze their channel activity by size exclusion of solutes or by ion conductance measurements. Both analyses establish the channel characteristics that represent the in vitro identity of a porin. Channel activity of porin was first described by Nakae in 1976 ***(3).*** Further studies demonstrated either selectivity or specificity, depending on the porin considered. The size exclusion limit is in direct relationship to the channel diameter, whereas the conductance speaks to its ionic selectivity (cationic or anionic). Taken together, these data may corroborate the structure of the porin deduced from crystalographic analysis, especially the structure of the channel. The understanding of porin structures and their relationship with other bacterial components may help in the development of new purification methods, especially when applied to new unknown porins.

The outer membrane of Gram-negative bacteria is a biologically unusual membrane in that it is a fully asymmetric lipid bilayer. The inner leaflet is composed mainly of phospholipids, whereas the outer one contains lipopolysaccharide (LPS) as the unique lipid species. This molecule is composed of the lipid A region, which is inserted into the membrane, the core region, and a hydrophilic oligosaccharide motif with a structure depending on species and strains ***(4).***

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The porins are, for the most part, assembled as homotrimeric structures, forming three independent and identical channels. After cleavage of the signal peptide the trimers are assembled during the export pathway. Numerous and very efficient interactions between the three monomers allow the trimer to be generally stable even in high-detergent concentrations ***(2)***. In several cases, however, no trimeric form can be evidenced, which may suggest a simpler way of assembly into the outer membrane.

In order to assemble its outer membrane, a Gram-negative bacteria encounter two main difficulties: 1) the movement of a membrane protein across the aqueous periplasmic space; and 2) the integration of the protein into an asymmetric membrane with a very hydrophobic outer part. The outer membrane assembly process is favored by two major structural properties of porins. First, the primary sequence analysis of porins does not show any hydrophobic stretch of amino acid residues, which differentiates them clearly from typical membrane proteins such as inner membrane proteins of Gram-negative bacteria ***(5)***. Second, crystalographic analysis of porins demonstrated an asymmetric organization of the molecules through the membrane. From the periplasmic side to the outer side of the membrane, one can found three distinct structural regions within porins: short periplasmic loops that are able to interact with the peptidoglycan layer, a long transmembrane barrel with a hydrophobic surface, and long external hydrophilic loops that are able to interact with the oligo-saccharide part of the LPS ***(6–9)***.

The three-dimensional structural studies also demonstrated how a hydrophilic polypeptide could be arranged in order to interact with a lipid bilayer. The major transmembrane domain is comprised of antiparallel β-sheets, which expose their hydrophobic amino acid residues to the lipidic membrane, thus forming the β-barrel, whereas the other residues are involved in the sheet to sheet interactions. Such numerous bounds may explain the unusual stability of these proteins.

Moreover, in the case of trimeric porins, interactions between monomers involve hydrophobic bonds associated to hydrogen bonds. In several cases, divalent cations also reinforce the trimeric structure.

In the Gram-negative rood *Campylobacter jejuni* (*C. jejuni*), the major outer membrane protein (MOMP) was first considered as a monomeric porin by Huyer et al. ***(10)***. We further demonstrated that, when mild solubilization conditions were applied for the purification, this protein behaved as a typical trimeric porin ***(11,12)***, thus demonstrating that purification conditions may dramatically affect the in vitro properties of such a molecule. MOMP was then described at the in vitro functional level as an OmpC like porin, one of the two major porins of *Escherichia coli* (*E. coli*) ***(13)***. At the structural level, however, molecular modeling of MOMP suggested an analogy with the family of sugar specific porins ***(14)***. More recently, we identified a second pore forming protein

in *Campylobacter*. This new porin, named Omp50 according to its molecular weight, showed neither sequence homology nor functional properties of already known porins ***(15)***. Structural data may help in the understanding of its role in the functional adaptation of the outer membrane of *C. jejuni*.

The method described in this chapter was first developed to purify the MOMP of *Campylobacter* ***(11)*** and then applied to other outer membrane porins of the bacteria, such as the recently identified Omp50 ***(15)***. The method follows four successive steps:

1. culture and preparation of bacteria;
2. membrane preparation by ultracentrifugation;
3. selective extraction of membrane proteins;
4. ion exchange chromatography and chromatofocusing.

2. Materials

2.1. Culture and Preparation of Bacteria

1. Muller–Hinton media supplemented with 5% sheep blood (Biomérieux).
2. Columbia Agar (Biomérieux) plates stored at 4°C for less than 1 wk.
3. 2YT medium: 10 g/L Bactotryptone, 16 g/L Yeast extract, and 10 g/L NaCl.
4. TE buffer pH 7.2: 10 m*M* Tris-HCl, pH 7.2, 1 m*M* ethylenediamine tetraacetic acid (EDTA), autoclaved, and stored at room temperature.
5. Glycine-HCl: 200 m*M* glycine, pH 2.2, stored at 4°C for less than 1 wk.
6. 100 m*M* Tris-HCl pH 7.2, and 10 m*M* Tris-HCl, pH 7.2, autoclaved, and stored at room temperature.

2.2. Membrane Preparation

1. Sonicator apparatus.
2. High-speed centrifuge.
3. Ultracentrifuge.

2.3. Selective Extraction of Membrane Proteins

1. Sodium lauryl sarcosinate 0.1% w/v in 10 m*M* Tris-HCl, pH 7.2.
2. Octyl-POE (Bachem AG, Bubendorf, Switzerland). Dilution of Octyl-POE in buffer must be prepared extemporaneously.
3. 20 m*M* sodium phosphate buffer pH 7.4.
4. Extraction buffer: octyl-POE (1% v/v) in 20 m*M* sodium phosphate buffer (pH 7.4).

2.4. Chromatography

1. An Akta Explorer 10 equipped with a MonoQ HR 10/10 column and a MonoP HR 5/20 from Amersham Biosciences were used for the ion exchange chromatography and the chromatofocusing, respectively.
2. Buffer A: NaPi pH 7.4, 0.6% Octyl-POE, 10 m*M* NaCl.
3. Buffer B: NaPi pH 7.4, 0.6% Octyl-POE, 1.0 *M* NaCl.

4. Equilibration buffer: 25 mM histidine, 10 m*M* NaCl, 0.6% octyl-POE, pH 6.0.
5. Elution buffer: Polybuffer 74 (Amersham Biosciences) 1/13 dilution, 100 m*M* NaCl, 0.6 % octyl-POE, pH 4.0 (*see* **Note 1**).

3. Methods

3.1. Culture and Bacteria Preparation

Because the Omp50 porin is not produced in *Campylobacter coli* (*C. coli*) ***(15)*** we used a *C. jejuni* strain to carry out the purification. *Campylobacter* was cultivated in microaerobic conditions at 42°C for all the experiments.

1. The *C. jejuni* strain is inoculated onto a blood Agar plate and cultivated for 24 h at 42°C.
2. The bacteria are then recovered in 1 mL of 2YT medium and 4 Columbia Agar plates supplemented with Campylosel (BioMérieux) are inoculated with this suspension (150 µL on each plate) and cultivated for about 48 h.
3. Each plate is then flooded with 2 mL of 2YT and the bacterial suspensions are pooled and inoculated onto 40 plates of Columbia Agar (150 µL on each plate) and cultivated for 48 h at 42°C.
4. Each plate is flooded with 5 mL of TE and incubated on a rotary agitator for 20 min at room temperature.
5. The bacterial suspension is then recovered, and each plate was rinsed with 1 mL of 2YT. At this point, the OD at 600 nm should be about 3.0.
6. The bacteria are recovered by centrifugation at 8000*g* for 30 min, resuspended in an equal volume of ice cold glycine-HCl, and incubated for 20 min at 4°C under agitation.
7. Washed bacteria are pelleted by centrifugation at 8000*g* for 30 min, washed once with 100 m*M* Tris-HCl (pH 7.2), and resuspended in 120 mL of 10 m*M* Tris-HCl (pH 7.2). Samples can be frozen at this step until used (*see* **Note 2**).

2.2. Membrane Preparation

1. The bacterial suspension is sonicated on ice 10 times for 2 min with a resting time of 2 min between each period in order to avoid overheating. The temperature is checked regularly and resting time should be lengthened if necessary.
2. Unbroken bacteria are recovered by centrifugation (8000*g* 30 min) and the supernatant was kept on ice.
3. The bacterial pellet is then resuspended with 80 mL of 10 m*M* Tris-HCl (pH 7.2) and a second run of 10 sonications was performed. Cell debris is removed by centrifugation (8000*g* 30 min).
4. The two supernatants are pooled and a final centrifugation was performed (8000*g* 30 min) to ensure that all cell debris was removed.
5. Membrane vesicles are then recovered by ultracentrifugation 100,000*g* 1 h at 4°C); the supernatant containing soluble proteins was discarded.

2.3. Selective Extraction of Membrane Proteins

1. The membrane pellet is resuspended in 180 mL of ice cold sodium lauryl sarcosinate (0.1% w/v in 10 m*M* Tris-HCl, pH 7.2) and incubated for 20 min at 4°C under agitation to solubilize the inner membrane proteins.
2. Outer membrane vesicles are recovered by ultracentrifugation (100,000*g* 1 h at 4°C).
3. Selective extraction of integral outer membrane proteins is performed by resuspension in octyl-POE (1% v/v) in 20 m*M* sodium phosphate buffer (pH 7.4). Vesicles are gently resuspended in 120 mL of ice cold extraction buffer and incubated for 30 min under agitation at 4°C.
4. The insoluble residue is removed from the solubilized proteins by ultracentrifugation (100,000*g* 1 h at 4°C), and the supernatant was kept on ice.
5. The pellet is reextracted once with 60 mL of extraction buffer and centrifuged.
6. The supernatants are pooled, divided into 45-mL aliquots, and frozen at −20°C until used.

2.4. Chromatography

2.4.1. Ion Exchange Chromatography

Classical anion exchange chromatography was used in this step with a flow rate of 2 mL/min. Conductivity and OD at 280 nm were checked during all the chromatography steps (*see* **Note 3**).

1. A MonoQ HR ion exchange column is equilibrated with column buffer A.
2. A 45-mL aliquot of solubilized protein is thawed and centrifuged at 10,000*g* for 20 min.
3. The supernatant is loaded onto the column, and the column is washed with two column volumes (CV) of buffer A (*see* **Note 3**).
4. Proteins are eluted using a linear gradient (12 CV) from 10 m*M* NaCl to 1 *M* NaCl. Notice that the OD 280 nm and conductivity must be at the basal level before starting the gradient.

As shown in **Fig. 1**, two major peaks were identified on the chromatogram. Because the extraction procedure is very selective there were not many unbound proteins (low OD 280 nm) during the loading step and only a very low level of contaminants was detected on the chromatogram. At this step, the two major peaks correspond to the Omp50 and to the MOMP protein of *Campylobacter* (peaks 1 and 2, respectively). Ion exchange was repeated four times and the fractions corresponding to Omp50 and MOMP were pooled.

2.4.2. Chromatofocusing

Omp50 was eluted at about 300 m*M* NaCl during the ion exchange chromatography (*see* **Fig. 1**). In order to bind the protein onto the Mono-P column,

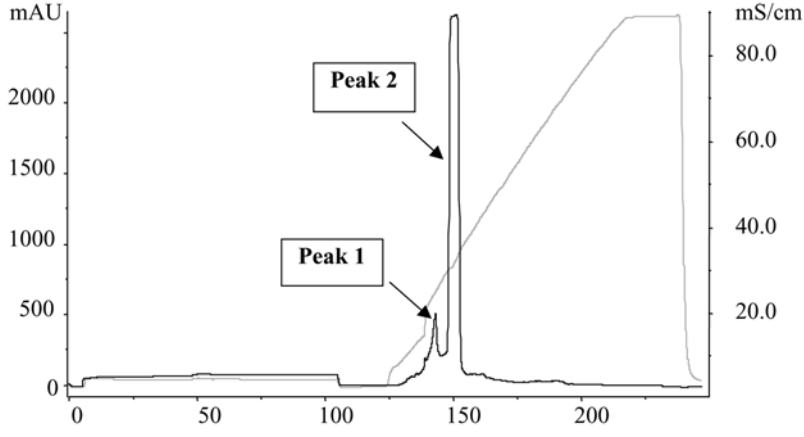

Fig. 1. Ion exchange chromatography of outer membrane proteins from *C. jejuni*. 45 mL of Octyl-POE extract were loaded onto the Mono-Q column. OD 280 nm is indicated on the left (mAU and black line) and conductimetry on the right in milliSiemens/cm (mS/cm and gray line). The volume is indicated in mL on the abscissa.

the salt concentration must be lowered to avoid competition with the protein. We chose dilution instead of dialysis, which is time and detergent consuming and which might induce loss of material.

1. The pooled fractions are thus diluted into sodium phosphate buffer (NaPi pH 7.4) supplemented with Octyl-POE (0.6%).
2. The Mono-P column is equilibrated with the same buffer containing 10 m*M* NaCl (Buffer A) before loading at a flow rate of 0.5 mL/min; this flow rate was maintained during all the chromatofocusing steps.
3. After loading and extensive washing, the buffer is changed to the equilibration buffer (25 m*M* histidine, 10 m*M* NaCl, 0.6% Octyl-POE, pH 6.0) until the pH and OD 280 nm reached their expected values (6.0 and about 0, respectively).
4. Elution was then performed with Polybuffer 74, and 1-mL fractions were collected. The pH and OD at 280 nm were checked during the elution process. As shown in **Fig. 2**, no contaminants were detected, however, several repeated experiments showed that after the ion exchange chromatography step, the protein solution was contaminated with a large amount of lipopolysaccharide, which was not detected by monitoring the optical density at 280 nm. After chromatofocusing, the LPS was undetectable by specific silver nitrate staining after Sodium dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4. Notes

1. The dilution of Polybuffer is the maximal dilution recommended by the manufacturer, which allowed us to obtain a longer pH gradient. Moreover, NaCl was added

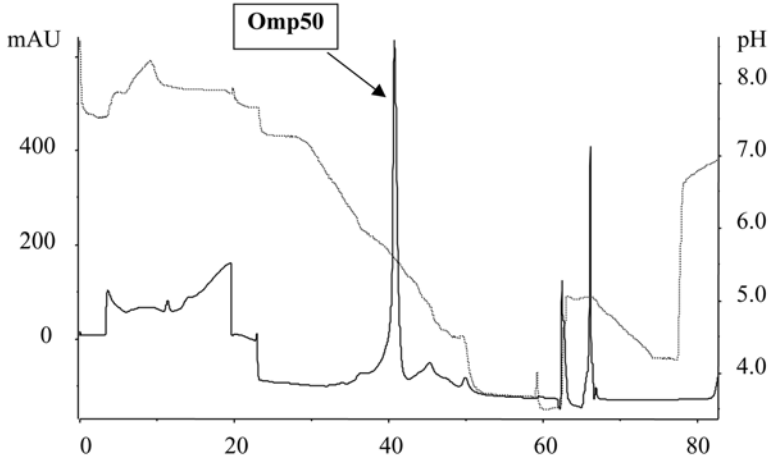

Fig. 2. Chromatofocusing of the pooled fractions corresponding to peak 1 of **Fig. 1**. OD 280 nm is indicated on the left (mAU and black line), pH is indicated on the right (dotted line). The volume is indicated in mL on the abscissa.

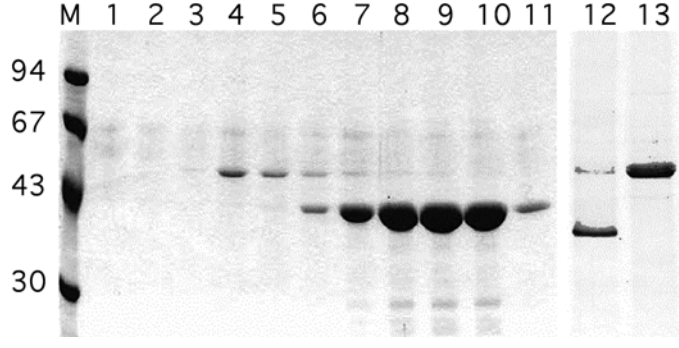

Fig. 3. SDS-PAGE electrophoresis of samples from chromatography. *M*, molecular mass standards (molecular weight are indicated on the left in Kilodalton); lanes 1 to 11 selected fractions from the ion exchange chromatography; lanes 12 and 13, pure Omp50 protein after chromatofocusing. Lanes 3 to 6 and 7 to 11 correspond to peaks 1 and 2 from **Fig. 1**, respectively. Samples were heat denatured before loading, except for lane 12, which was treated in sample buffer at room temperature allowing us to evidence the peculiar apparent molecular weight of Omp50 in these conditions (*see* **ref. *15***, for details).

into the elution buffer and its concentration (100 m*M*) was determined experimentally in order to favor elution of the porin.

2. A Potter crusher was used in order to facilitate all the resuspension steps.

3. Octyl-POE was included in each buffer and 10 m*M* NaCl was also added in the equilibration buffer in order improve protein solubilization by detergent micelles and column loading.

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12

Purification of Porins from *Mycobacterium smegmatis*

Christian Heinz, Eva Roth, and Michael Niederweis

1. Introduction

Mycobacteria possess a unique cell wall that mainly consists of a copolymer of peptidoglycan, arabinogalactan, and mycolic acids ***(1)***. The mycolic acids constitute the inner layer of an outer membrane with an exceptionally low fluidity ***(2)*** and permeability for hydrophilic compounds ***(3,4)***. Porins are water-filled channel proteins that mediate the transport of hydrophilic nutrients such as sugars and amino acids across the outer membrane of Gram-negative bacteria ***(5,6)***. A decade ago, channel proteins functionally similar to the porins of Gram-negative bacteria were discovered in mycobacteria ***(7,8)***.

MspA was the first porin identified in *Mycobacterium smegmatis* ***(9)***, a fast-growing, nonpathogenic, saprophytic bacterium that is often used as a model system for mycobacteria. MspA is an oligomer with an apparent molecular mass of 100 kDa in denaturing polyacrylamide gels. It retains its capability to form open channels in lipid bilayers with a conductivity of 4.6 nS, even if exposed to heat or a denaturing agent like SDS. MspA dissociates into 20-kDa monomers of 184 amino acids after heating in the presence of organic solvents such as dimethy/sulfoxide (DMSO) and at very high temperatures ***(9)***.

Recent studies revealed that MspA belongs to a family of four very similar porins in *M.smegmatis*. Deletion of *mspA* drastically reduced the uptake of glucose, showing that MspA provides the main hydrophilic pathway through the cell wall of *M.smegmatis* ***(4)***.

Because there is no homology of the Msp proteins to any other protein in the database, MspA was thought to be the prototype of a new class of porins. Up to now, the main porin class is defined by the trimeric porins of Gram-negative bacteria, where each monomer forms one channel ***(6)***. However, little is known

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about structural, biochemical, and functional properties of MspA in particular and mycobacterial porins in general. So far, only tiny amounts of these proteins, in the range of 10 μg per liter of culture, could be isolated from *M. smegmatis* using preparative gel electrophoresis ***(9)***. This raised the demand for a better purification method. In this chapter, we describe a rapid and efficient purification method for porins from *M. smegmatis*, which yields 20-fold higher amounts of pure and active MspA compared to previous protocols. This method is based on the selective extraction of MspA from cells of *M. smegmatis* using nonionic detergents at high temperatures ***(10)***. Here, we show that destruction of the cells prior to extraction and use of SDS did not increase the amount of extracted MspA. Purification of MspA to homogeneity is achieved by anion exchange chromatography and subsequent gel filtration. We show further that using this procedure, MspC, and most likely other porins of the Msp family, are as efficiently purified as MspA.

1.1. Selective Extraction of MspA

The first step in purification of membrane proteins is their extraction from the membrane. This is normally achieved by treating whole cells or isolated outer membranes with mild detergents at room temperature ***(11)***. Cell extracts of *M. smegmatis* obtained by this method showed pore forming activity, but contained only minor amounts of MspA and many contaminating proteins ***(10)***. Active MspA was also extracted from cells with organic solvents, but the yield was low ***(9)***. However, boiling of *M. smegmatis* cells with nonionic detergents yielded not only increased amounts of MspA, but removed most of the contaminating proteins ***(10)***. Best extraction results were achieved by using nonionic detergents at temperatures above 90°C. These detergent extracts contained about 85% pure MspA with a high channel-forming activity ***(10)***. Thus, by exploiting the heat resistance of MspA, an efficient and selective extraction procedure was found (*see* **Fig. 1A,** lanes 6–10). We assume that extended boiling of the cells is a major determinant of the exceptional selectivity of this method, because most likely other proteins in this the extract usually denature and easily precipitate under those conditions in contrast to MspA. However, it was not known, whether MspA was quantitatively extracted from whole cells by this procedure. Therefore, we compared the extraction of MspA from entire and lysed cells with detergents in dependence of the boiling time. The amount of MspA extracted with n-octylpolyoxyethylene (OPOE) from lysed cells increased with incubation time and reached a plateau value after 30 min (*see* **Fig. 1B**). Surprisingly, a similar amount of MspA was extracted from whole cells already after 5 min indicating that cell lysis before extraction did not improve the yield of extracted MspA and was not selective anymore (**Fig. 1A,** lanes 1–5). Moreover, no additional MspA was solubilized by OPOE from the

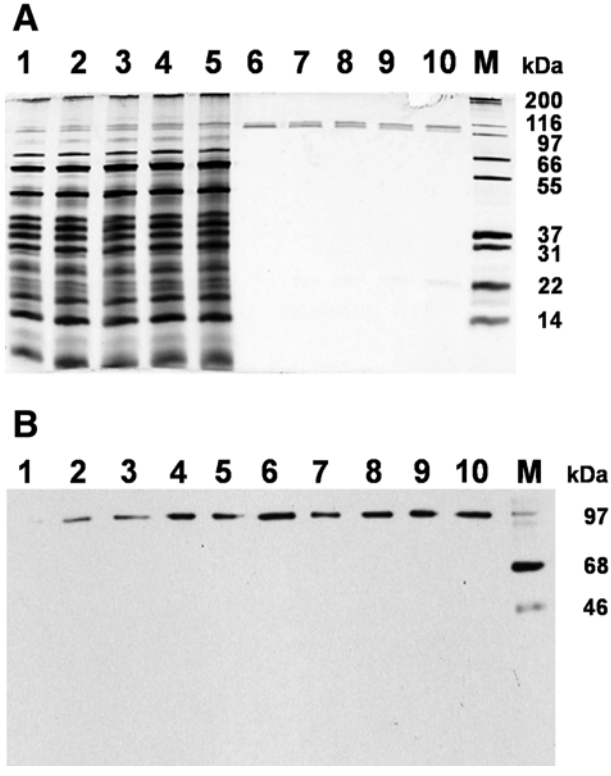

Fig. 1. Analysis of MspA extraction with n-octylpolyoxyethylene (OPOE). The samples were incubated at room temperature with 5 μL loading buffer for 10 min. **(A)** 10 % polyacrylamide gel stained with silver. Lanes 1–5, 9 μL each of broken cell extracts treated with 0.5 % OPOE; incubation times were 5, 10, 20, 30, and 60 min; lanes 6–10, 8 μL each of whole cell extracts treated with 0.5% OPOE; incubation times were 5, 10, 20, 30, and 60 min; lane M, 4 μL protein mass marker (Mark12). **(B)** Western blot analysis. The proteins were separated on a denaturing 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Proteins were visualized using the MspA antiserum ***(9)*** and a chemiluminescence reaction. Lanes were loaded as in Fig. 1A, but with less protein. Lanes 1–5, 2 μL extracts diluted 100-fold with POP05 buffer; lanes 6–10, 2 μL of extracts diluted 250-fold with POP05 buffer. Lane M, 1μL ECL Protein Molecular Weight Markers (Amersham-Pharmacia).

lysed cells indicating that no MspA was located in the cytoplasm. Sodium dodecyl sulfate (SDS) solubilized more proteins from lysed cells than OPOE (*see* **Fig. 2A**). However, immunoblotting revealed that only tiny amounts of MspA were extracted with SDS from the cell fragments compared to extraction of whole cells with OPOE (*see* **Fig. 2B**). This result confirmed earlier observa-

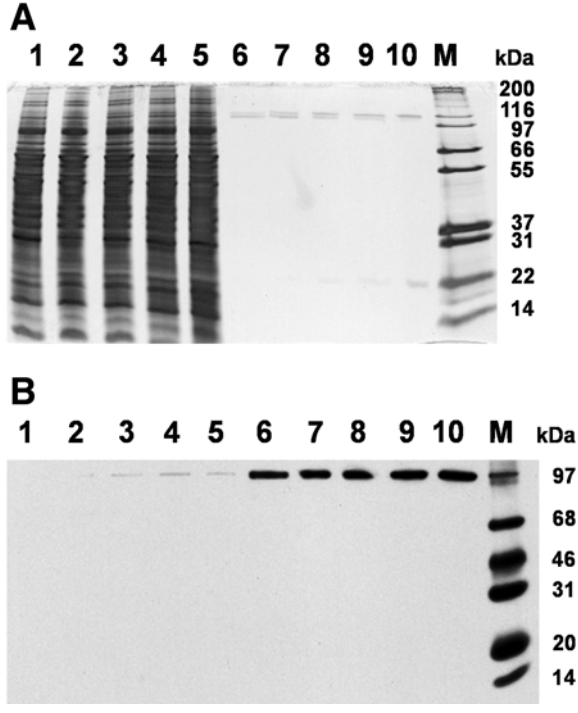

Fig. 2. Analysis of MspA extraction with SDS. The samples were incubated at room temperature with 5 μL loading buffer for 10 min. **(A)** 10 % polyacrylamide gel stained with silver. Lanes 1–5, broken cell extracts treated with 1% SDS with 750 ng protein per lane; incubation times were 5, 10, 20, 30, and 60 min; lanes 6–10; 2 μL of whole cell extracts treated with 0.5 % OPOE; incubation times were 5, 10, 20, 30, and 60 min; lane M, 4 μL protein mass marker (Mark12). **(B)** Western blot analysis. The proteins were separated on a denaturing 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Proteins were visualized using the MspA antiserum ***(9)*** and a chemiluminescence reaction. Lanes were loaded as in **Fig. 2A,** but with less protein. Lanes 1–5, 1.5 ng protein per lane; lanes 6–10, 2 μL extracts diluted 500-fold with POP05 buffer; lane M, 1 μL ECL Protein Molecular Weight Markers (Amersham-Pharmacia).

tions that the choice of detergent is critically important and that extraction efficiency does not correlate with solubilization strength ***(10)***. It is concluded that the selective extraction procedure as described by Heinz and Niederweis ***(10)*** is the best initial step for the purification of MspA from *M. smegmatis*. Using this procedure, the porin MspC is as efficiently extracted from *M. smegmatis* as MspA ***(4)***. Because MspC and the two other known porins of the Msp family, MspB and MspD, are closely related to MspA, we assume that selective extrac-

tion from whole cells at high temperatures using nonionic detergents is a general method for the isolation of porins of the Msp class from *M. smegmatis*.

1.2. Purification of MspA

Ion exchange chromatography is widely used for protein purification ***(12)*** and has the advantage of concentrating the protein of interest. However, this excludes the use of detergents with a charge opposite to that of the column material for purification of membrane proteins. In this regard, nonionic detergents such as OPOE are preferable for the initial solubilization step.

MspA was precipitated by acetone from the crude extract to remove lipids and to concentrate the sample. This step did not lead to a detectable loss of protein (*see* **Fig. 3B,** lane 2). Then, MspA was purified to apparent homogeneity in a single step by anion-exchange chromatography (*see* **Fig. 3B**). The major part of contaminating proteins eluted at NaCl concentrations below 700m*M* from the anion exchange column (*see* **Fig. 3A,B,** lanes 4–6). MspA eluted at a concentration of 0.82 *M* NaCl, which is in accordance with previous results ***(10)***. It should be noted that MspC, a porin isolated from an MspA-deficient mutant ***(4)***, eluted at a concentration of 1.3 *M* NaCl and is, therefore, completely separated from MspA. These results indicated that MspA, when purified by the method described here, is neither contaminated by MspC nor by other proteins. For buffer and detergent exchange, a gel filtration chromatography may be performed (*see* **Note 7**).

2. Materials

2.1. Bacterial Strain

M. smegmatis strain mc^2155: A high-frequency transformation derivative of mc^26.

2.2. Growth and Preparation of M. smegmatis *mc²155 Cells*

1. Middlebrook 7H10 agar (Difco): Dissolve 19 g of powdered 7H10 medium in 995 mL deionized water and add 5 mL of 100% glycerol. Autoclave for 15 min, mix well, and pour in standard plastic Petri dishes.
2. Middlebrook 7H9 Broth (Difco): Dissolve 4.7 g broth base in 994.2 mL deionized water. Add 2.5 mL 20% Tween-80 and 3.3 mL 60% glycerol, mix well, and autoclave for 10 min.
3. Phosphate-buffered saline (PBS): 100 m*M* Na_2HPO_4/NaH_2PO_4, 0.1 m*M* Na_2EDTA, 150m*M* NaCl, pH 7.0.

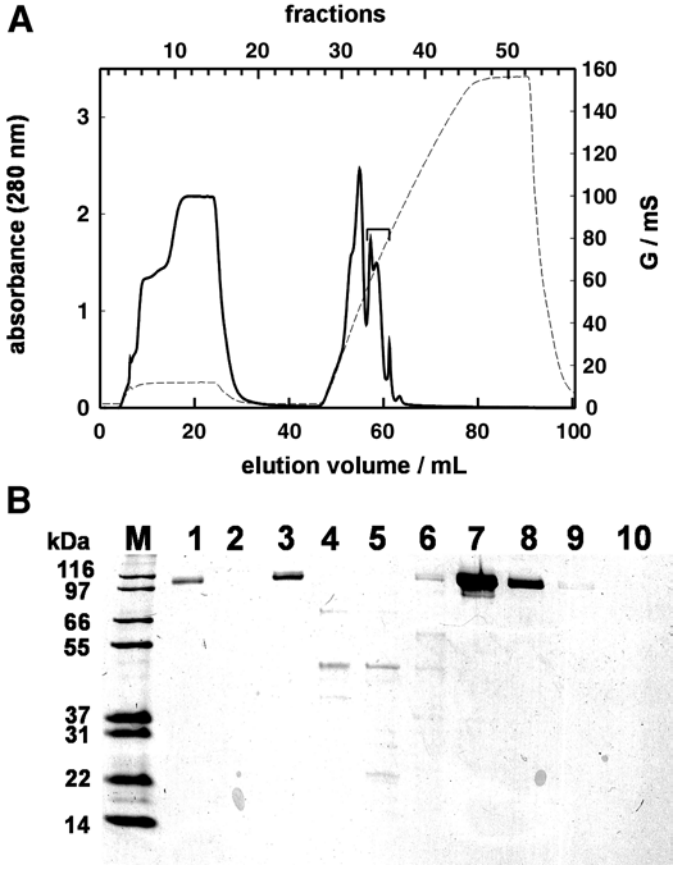

Fig. 3. Purification of MspA by anion exchange chromatography. **(A)** The solid line represents the absorbance at 280 nm, the dashed line the conductivity. Elution with a linear gradient from 0 to 2 *M* NaCl eluted MspA between 0.70 and 0.98 *M* NaCl with the peak at 0.82 *M* NaCl (marked with a bracket). **(B)** Analysis of the fractions of anion exchange chromatography in a 10% polyacryamide gel stained with Coomassie. The samples were incubated at room temperature with 5 μL loading buffer for 10 min. Lane M, molecular mass marker (Mark12). Lane 1, 10μL whole cell extract; lane 2, 10μL supernatant after precipitation with acetone; lane 3, 10μL of the solution containing the dissolved pellet after precipitation with acetone; lanes 4–10, 15 μL of the fractions 35–41 of the anion exchange chromatography. Fractions 38 and 39 contained the highest amount of MspA (lanes 7 and 8) and were pooled.

2.3. Polyacrylamide-Gel Electrophoresis (PAGE)

1. Anode buffer: 0.2 *M* Tris-HCl, pH 8.9.
2. Cathode buffer: 0.1 *M* Tris, 0.1 *M* Tricine, 0.1% SDS, pH 8.25 (no adjustment of the pH).
3. Gel buffer: 3.0 *M* Tris, pH 8.45 (adjusted with HCl).
4. Acrylamide-*bis*-acrylamide mixture "Gel 30" (Roth, Karlsruhe, Germany): 30% acrylamide, 0.8% bis-acrylamide.
5. Loading buffer: 4X buffer is 140 m*M* Tris-HCl pH 7.5, 30% (w/v) glycerol, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue.

2.4. Silver Staining of Polyacrylamide Gels

1. Fixing solution: 40% (v/v) ethanol, 10% (v/v) acetic acid.
2. Sensitizing solution: 0.83 *M* sodium acetate, 0.2% (w/v) sodium thiosulfate, 30% (v/v) ethanol, 0.13% (v/v) glutardialdehyde.
3. Silver solution: 0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde.
4. Developing solution: 0.24 *M* sodium carbonate, 0.007% (v/v) formaldehyde.
5. Stop solution: 0.04 *M* Na_2EDTA.
6. Preserving solution: 30% (v/v) ethanol, 4% (v/v) glycerol.

2.5. Coomassie Staining of Polyacrylamide Gels

1. Fixing solution: 40% (v/v) ethanol, 10% (v/v) acetic acid.
2. Coomassie solution: 0.1% (w/v) PhastGel Blue R (Amersham Biosciences), 30% methanol, 10% acetic acid; prepare solution according to manufacturers instructions.
3. Preserving solution: 30% (v/v) ethanol, 4% (v/v) glycerol.

2.6. Western Blotting

1. Nitrocellulose membrane "Protran BA 85" (Schleicher & Schuell).
2. Whatman paper 3MM CHR (Whatman).
3. Transfer buffer: 10 m*M* $NaHCO_3$, 3 m*M* Na_2CO_3, 20% (v/v) methanol.
4. 10X TBST: 100 m*M* Tris-HCl, pH 8, 1.5 *M* NaCl, 0.5% (v/v) Tween-20.
5. Blocking solution: 5% (w/v) milk powder in 1X TBST.
6. Primary antibody: 1% (w/v) milk powder in 1X TBST, 1:2000 (v/v) polyclonal antibody #813 against MspA.
7. Secondary antibody: 1X TBST, 1:5000 (v/v) antirabbit HRP, 1:2000 (v/v) streptavidin-HRP conjugate (Amersham).
8. Detection: ECL plus chemoluminescence system (Amersham).

2.7. Preparation of Whole Cell Extracts

1. 3X PEN buffer: 300 m*M* Na_2HPO_4, 200 m*M* NaH_2PO_4, 450 m*M* NaCl, 0.3 m*M* EDTA, pH 6.5.
2. POP05 buffer: 1X PEN (pH 6.5), 0.5% (m/v) n-octylpolyoxyethylene (OPOE, Bachem).

2.8. Preparation of Broken Cell Extracts

1. Bead-beater cell disruptor: FastPrep FP120 (dianova, Hamburg).
2. 1X PEN buffer.
3. Glass beads; diameter 0.10–0.11 mm (Braun, Melsungen)
4. Detergent of choice: e.g., OPOE.

2.9. Chromatographic Purification of MspA

1. HPLC system: e.g., Biocad 700E (Perkin-Elmer)
2. Anion exchange column: POROS 20HQ with bed volume of 1.7 mL (Perkin-Elmer).
3. Gel filtration column: G4000PW_{XL} with bed volume of 14.3 mL (Tosoh Biosep).
4. AOP05 buffer: 25 m*M* HEPES, 10 m*M* NaCl, 0.5% (m/v) OPOE, pH 7.5.
5. BOP05 buffer: 25 m*M* HEPES, 2 *M* NaCl, 0.5% (m/v) OPOE, pH 7.5.
6. NaPOP buffer: 25 m*M* Na_2HPO_4/NaH_2PO_4, 0.5% (m/v) OPOE, pH 7.5.

3. Methods

3.1. Growth and Preparation of Mycobacterium smegmatis *mc²155 Cells*

1. Streak out from a frozen stock culture of *M. smegmatis* mc^2155 on a 7H10 plate and incubate for 3 d at 37°C.
2. Inoculate 5 mL of Middlebrook 7H9 medium with some colonies taken from plate. Incubate at 37°C with shaking (190 rpm) for 2 d, at which time the culture should be turbid.
3. Inoculate 1 L of Middlebrook 7H9 medium with 2 mL of the preculture and incubate at 37°C with shaking (190 rpm) for 2 d (*see* **Note 1**).
4. Harvest the cells by centrifuging for 15 min at 4°C at 11,000*g*.
5. Wash the pellet by resuspending in 25 mL PBS buffer. Sediment the cells by centrifuging for 15 min at 4°C at 27,000*g*. The cells can now be used directly or can be stored at –20°C.

3.2. Preparation of Whole Cell Extracts (Preparative Scale)

1. Resuspend 10 g of *M. smegmatis* cells (wet weight) in 35 mL POP05 buffer and boil them under stirring for 30 min (*see* **Note 2**).
2. Cool down the cell suspension on ice for 10 min.
3. Centrifuge the mixture for 15 min at 4°C at 27,000g. The supernatant (crude extract) can now be used directly for further purification or can be stored at –20°C.

3.3. Preparation of Broken Cell Extracts (Analytical Scale)

1. Resuspend 10 mg *M. smegmatis* cells (wet weight) in 35 μL POP05 buffer.
2. Mix the cell suspension with suspended glass beads (volume ratio 3:1).
3. Disrupt the cells with bead-beater cell disruptor FastPrep FP120 (stage 6, 2 × 20 s, *see* **Note 3**).
4. Adjust the samples to a final concentration of 1% SDS or a final concentration of 0.5% OPOE (*see* **Note 4**).

5. Boil the samples for 30 min.
6. Cool down the suspension on ice for 10 min.
7. Centrifuge the suspension for 15 min at 4°C at 27,000*g*. The supernatant can now be used directly or can be stored at −20°C.

3.4. Chromatographic Purification

3.4.1. Anion Exchange Chromatography

1. Mix the crude extract with an equal volume of cold acetone and cool the mixture on ice for 1 h (*see* **Note 5**).
2. Centrifuge the mixture for 15 min at 4°C at 7700*g*.
3. Dissolve the resulting pellet in 20 mL AOP05 buffer and filtrate through a 5-μm filter (*see* **Note 6**).
4. Perform following steps on the HPLC system:
   - Load the regenerated anion exchange column 20HQ wiht the sample and start fraction collection.
   - Wash with 13 mL AOP05 buffer.
   - Apply gradient from 100% AOP05 buffer to 100% BOP05 buffer over 33 mL.
   - Hold 100% BOP05 buffer over 13 mL.
5. Analyze the collected fractions by PAGE.

3.4.2. Gel Filtration (see ***Note 7***)

1. Pool the fractions with highest MspA content after anion exchange chromatography.
2. Add the same volume of cold acetone, mix gently, and incubate the mixture for at least 30 min on ice.
3. Centrifuge for 15 min at 4°C at 7700*g*.
4. Dissolve the pellet in 600 μL NaPOP buffer and centrifuge for 5 min at 16,000*g* at room temperature.
5. Perform following steps on the HPLC system:
   - Load the equilibrated gel filtration column G4000PW$_{XL}$ with the supernatant and start fraction collection.
   - Elute with 29 mL NaPOP buffer.
6. Analyze the collected fractions by PAGE and pool pure fractions.
7. Determine protein concentration using the BCA assay (Pierce).
8. Store MspA at room temperature (*see* **Note 8**).

3.5. Polyacrylamide-Gel Electrophoresis (PAGE)

Polyacrylamide gels are prepared according to the method of Schaegger and Jagow ***(13)***. For most applications a polyacrylamide concentration of 10% is appropriate.

1. Incubate protein containing samples in loading buffer for 10 min at room temperature.
2. Load the samples onto the gel and include a suitable protein marker (e.g., Mark12, NOVEX).

3. Run the gel at 120 V for 1.5 h, current should be about 70 mA (*see* **Note 9**).
4. After electrophoresis, stain the gel with silver or Coomassie or prepare the gel for Western blotting.

3.6. Western Blotting

Blotting procedure is performed using a standard protocol ***(14)***.

1. Equilibrate the PAA gel and the nitrocellulose membrane for 20 min by shaking in transfer buffer.
2. Assemble the gel and membrane according to the instructions of the manufacturer of the blotting chamber.
3. Fill the chamber with transfer buffer and apply a current 50 mA when blotting overnight or 150 mA when blotting for 1.5 h.
4. Treat the membrane after transfer with following solutions while shaking:
   - 1 h blocking solution.
   - 1 h primary antibody (e.g., anti-MspA serum #813).
   - 3 × 5 min. 1× TBST (washing step).
   - 1 h secondary antibody.
   - 3 × 5 min. 1× TBST (washing step).
5. Visualize the transferred protein according to the instructions of the manufacturer of the ECL plus chemolumiscence system.

3.7. Silver Staining of Polyacrylamide Gels

Staining is performed using a standard protocol ***(15)***.
Treat the polyacrylamide gel with following solutions while shaking:

1. 30 min fixing solution.
2. 30 min sensitizing solution.
3. 3 × 5 min water (*see* **Note 10**).
4. 20 min silver solution.
5. 2 × 1 min water.
6. 4 min developing solution.
7. 10 min stop solution.
8. 2 × 10 min water.
9. 20 min preserving solution.
10. drying of the gel (e.g., using the Dry Ease Kit, NOVEX)

3.8. Coomassie Staining of Polyacrylamide Gels

Treat the polyacrylamide gel with following solutions while shaking:

1. 30 min fixing solution.
2. 5–10 min Coomassie solution.
3. 3× destaining solution (*see* **Note 11**).

4. Notes

1. The color of the culture may vary from white to orange or brownish without any detectable influence on the quality of the cell extracts. If, however, a contamination of the culture with other bacteria is suspected, which might overgrow the relatively slow-growing *M. smegmatis*, a visual inspection of the culture for the typical mycobacterial cell aggregates or an examination of the bacteria under the microscope is recommended.
2. The time required for complete extraction of MspA varies depending on the sample volume. For example, complete extraction of MspA from 10 mg cells in 35 μL is already achieved after 5 min, whereas incubation for 30 min is needed for extraction of MspA from cells in a volume of 35 mL (preparative scale).
3. Incubate the cell suspension between the shaking intervals for some minutes on ice to avoid overheating.
4. The detergents should be added after the shaking intervals to prevent foaming.
5. If the precipitation step with acetone is omitted, MspA does not elute in a sharp peak. This could be caused by a higher amount of lipids in the sample. Unspecific binding of lipids to MspA might differentially influence its binding to the column.
6. Filtration through a 5-μm filter (e.g., Minisart, Sartorius) is strongly recommended to remove insoluble particles, which might clog the filters or the column of the HPLC system. Loss of protein by adsorption to the filter membrane was not observed.
7. MspA containing fractions are usually pure enough after anion exchange chromatography and do not require further purification. Gel filtration may be done for buffer exchange. The yield of pure MspA after gel filtration varies between 0.5 and 1 mg from 10 g cells (wet weight).
8. Purified MspA can be stored at room temperature and is stable in POP05 buffer for at least 6 mos without a detectable loss of channel-forming activity. Storage at –20°C is also possible, but diluted samples (<100 ng/μL) appear to lose activity. However, it should be noted that there is no reliable method to easily quantify the channel-forming activity of pore proteins. Therefore, storage conditions were not checked vigorously for their effect on activity on MspA.
9. Electrophoresis is stopped when the blue marker dye leaves the bottom of the gel.
10. Attention should be paid during the washing process that the gel does not adhere to the bottom of the Petri dish or similar vessels. Gels that stuck to surfaces during washing tend to develop a brownish background during the treatment with the silver solution.
11. Destaining should be performed until the blue background has vanished. This may take several hours depending on the time of the preceeding staining step and freshness of the Coomassie solution. Repeated changing and heating of the destaining solution accelerates the destaining procedure.

Acknowledgments

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13

Isolation of the Melanocortin 5 Receptor

From cDNA Sequence to Isolating an Integral Membrane Protein

Benjamin L. Clarke

1. Introduction

The family of melanocortin receptors (MCR) specifically binds peptide hormones derived from propiomelanocortin, including adrenocorticotropic hormone (ACTH) and alpha-melanocyte stimulating hormone (αMSH). These receptors are generally characterized as integral proteins having seven membrane-spanning domains and a short carboxyl tail on the cytosolic side, and encoded by intronless genes. Currently five different MCRs have been identified and cloned ***(1)***. The ligand-binding specificity and equilibrium-binding constants for a variety of natural and synthetic analogues of the melanocortins have been determined for each MCR. In general, this information has been obtained by measuring ligand binding to cell lines transfected to overexpress receptor mRNA. Successful transfection has generally been determined by reverse transcriptase-polymerase chain reaction (RT-PCR). A follow-up experiment invariably tests for ligand-specific stimulation of adenylyl cyclase activity to confirm that hormone binding stimulates a G-protein coupled receptor to dissociate from a $G_{\alpha s\text{-}GTP}$. These experiments have produced important information about binding and have begun the process toward developing both agonists and antagonists to MCR mediated activities. In addition, earlier cloning work has produced a valuable source of information concerning the inferred amino acid sequence based on the cDNA and information about probable tissue expression of these receptors. However, these studies do not give other important information concerning the functional size of these receptors and the putative presence of posttranslational modifications such as glycosyla-

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tion or phosphorylation. Also, these studies neither address the potential receptor internalization by endocytosis, nor the life-span of a receptor from *de novo* synthesis to senescence. To begin, productive studies on the physiochemical properties of the native receptor requires isolating the protein.

The following method describes a procedure used in our laboratory to isolate the MC5-R plus additional work to isolate the MC3 and MC4 receptors from rat brain tissue (Kroiss, Githu and Clarke; manuscript in preparation). The protocol takes advantage of cDNA data to generate polyclonal antisera that specifically recognize the MC5-R. Two short amino acid sequences were identified based on the inferred sequence data for the rat MC5-R ***(2)***. These sequences were taken from positions 1-20 and 298-307. Initially, rabbit antiserum was generated to the dodecamer sequence taken from the amino terminus end of MC5-R. The antiserum was used to screen for MC5-R expression in a variety of rat tissues using a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western protocol. A relatively large protein band was identified, which we had originally reported as 77,000 ($p77$) and specific to the antisera. These initial experiments showed the receptor to have an exceptionally large size compared to the expected size of 37,000, based on cDNA sequence information. Therefore, we took extra precaution to confirm the identity of the larger protein as MC5-R.

Different antisera were generated to a peptide sequence found toward the carboxyl terminus of MC5-R. The second antisera also identified the same protein band confirming the identified protein as authentic MC5-R by SDS-PAGE/Western. The amino terminus directed antisera was then used to immunoprecipitate MC5-R, which was in turn recognized by the carboxyl terminus directed antiserum in a subsequent SDS-PAGE/Western. Reversing the order to immunoprecipitate with antiserum to the carboxyl terminus and identifying the protein by SDS-PAGE/Western using antiserum to the amino terminus produced the same conclusion. Thus, isolated protein contained the same two antigenic sites that are expected for MC5-R. The identification of a larger than expected protein to represent MC5-R was surprising; the excess mass still presented an enigma. One direct approach was to check for N-linked carbohydrate. Immunoprecipitated protein was treated with protein N-linked glycosidase and demonstrated to have a reduced size at approx 35,000. Thus, glycosyl groups play an important role in the overall MC5-R structure. However, we do not know if the size difference is owing only to the carbohydrate moiety or if the glycosylation favors a multimeric complex.

Recent work in our laboratory has provided additional insight concerning the effective use of detergents to isolate the MC5 receptor. In general, we found that a mixture of an alkyl ionic and polyoxyethylene detergents, plus a bile salt gives the best results. *See* **ref. *3*** for a good introduction on detergents. Our

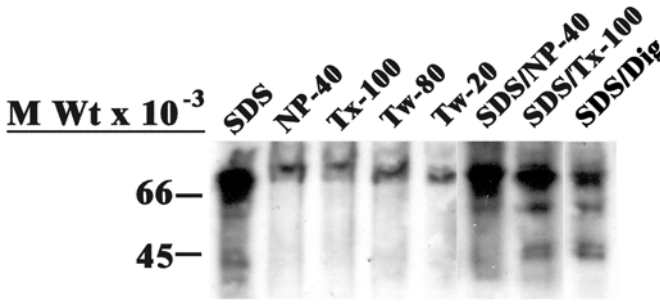

Fig. 1. Differential solubilization of lachrymal tissue alters MC5 detection. Lachrymal tissue was diced and homogenized in a phosphate buffered saline plus PIC1. The material was then divided into equal portions and mixed with different detergents to give a final concentration of 1.0 % for each detergent type and processed as described in **Subheading 3.1**. Each lane represents 2.5 μg of solubilized crude proteins separated on an 8% SDS-PAGE (0.5% *bis*-acrylamide cross-linking) resolving gel. The resolved proteins were electrophoretically transferred to nitrocellulose and probed for MC5-R using antibody directed to the carboxyl terminus.

usual detergent mixture consists of SDS, Triton X-100 plus deoxycholate, which is able to effectively isolate both the larger form of MC5, and a smaller size of MC5 at 45,000 (*see* **Fig. 1**). We also observed that a choice of the resolving gel used during the electrophoresis step alters the interpretation of the receptor size (*see* **Fig. 2**). Our current studies which uses an 8% gel and a 5% cross-linking gives the size of MC5-R at 68,000 and 45,000. The two observed sizes of MC5-R vary in expression in various rat tissues, both in abundance and relative distribution.

2. Materials

2.1. Reagents

1. Tissue Solubilization Buffer: First prepare a buffered saline solution by mixing 1.35 g NaCl, 0.4 g Trizma®-HCl (Tris-HCL), 0.15 g ethylenediaminetetraacetic acid (EDTA), and dissolve into 90 mL deionized water; adjust the pH to 7.4 and then expand the volume to 100 mL with additional deionized water. Tissue solubilization buffer is prepared by adding 1 g SDS, 1 mL Triton X-100, 0.5 g sodium deoxycholic acid, and 100 μL of PIC1 (*see* **Subheading 2.2.**). Bring the final volume up to 100 mL using deionized water. This buffer contains 250 m*M* sodium chloride, 25 m*M* Tris-HCL pH 7.4 containing 1% SDS, 1% Triton X-100, and 0.5% deoxycholate, 5 m*M* EDTA plus protease inhibitors.
2. Protease Inhibitor Cocktail: Protease inhibitor cocktail (PIC1) is made by dissolving the following compounds (Sigma-Aldrich Fine Chemicals) into 10 mL of a saline solution of aprotinin: 10 mg leupeptin, 20 mg antipain, and 100 mg benzamidine. A

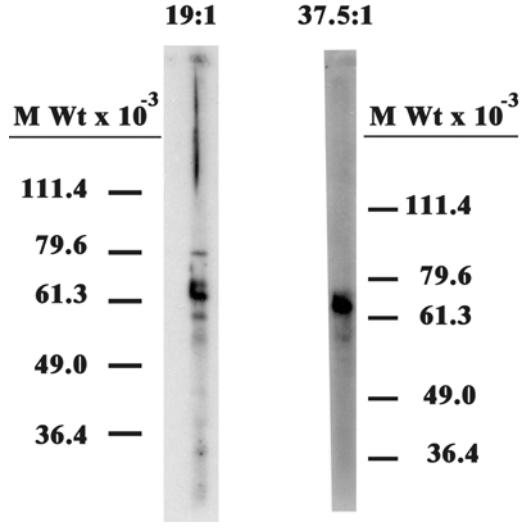

Fig. 2. Effect of the degree of gel cross-linking on protein resolution. Lachrymal tissue was solubilized as described in **Subheading 3.1.,** and 2.5 μg of solubilized crude proteins separated on an 8% SDS-PAGE. Two different types of cross-linking conditions were used to separate the crude proteins: 19:1 is 5% cross-linked, and 37.5:1 is 2.6% cross-linked.

second formulation is sometimes used if detergent solubilization is limiting to provide additional protection. Dissolving the following compounds in 10 mL of dimethyl sulfoxide (DMSO) makes PIC2: 10 mg chymostatin and 10 mg pepstatin. We use a combination of protease inhibitors in a 1000-X concentrated form; once diluted this PIC1 gives a final concentration of 1 μg/mL leupeptin, 2 μg/mL antipain, 10 μg/mL benzamidine, 10 μg/mL aprotonin. The PIC1 is relatively stable and is added to provide long-term protection. In some cases, we added a potent inhibitor of cysteinyl proteases to a final concentration of 1 m*M* phenylmethylsulfonyl fluoride (PMSF) from a stock solution of 100 m*M* in ethanol (*see* **Note 1**).

3. Electrophoresis Reagents: Our laboratory uses the Bio-Rad MiniGel® system. In general, any standard recipes for performing SDS-PAGE will work. However, close attention should be paid to the extent of cross-linking used for the gels, and that several different gel densities are tested to determine which gives the desired response; typically densities between 8–12% work fine. Readers who are unfamiliar with SDS-PAGE are suggested to read previously published methods ***(4)***. Also, the selection of size markers is critical and considerations such as efficiency of transfer during electroblotting, ease of discerning the different size markers, and a good size spread to allow for simple size estimation. Currently, our laboratory uses the BenchMark™ Standards from Gibco-BRL

(Cat. 10748-010, Life Technologies Inc., Gaithersburg, MD), which give good spacing between the different sizes plus a single reference band to orient the investigator to the gel.

4. Electroblot Transfer Buffer: Mix 3.03 g Trizma®-base, 14.4 g glycine in 500 mL deionized water, then add 200 mL of methanol, and adjust the final volume to 1 L with deionized water.
5. Membrane Washing Buffer: A 10X concentrate of phosphate buffered saline (PBS) is prepared containing 2 g potassium chloride, 80 g sodium chloride, 2 g potassium phosphate, and 11.5 g sodium phosphate dibasic in 1-L water at a final pH 7.4. Next, the concentrate is diluted 1:9 with distilled water and then Tween®-20 is added to a final concentration of 0.1%. Therefore, mix 100 mL concentrate with 900 deionized water and add 1 mL of Tween-20 to make a 0.1% PBST. The final solution is used to wash membranes.
6. Blocking Buffer: Dry milk (5 g) is added to 100 mL of PBST (*see* **step 5**) (*see* **Note 2**).
7. Primary Antibody cocktail: Rabbit polyclonal antiserum specific to MC5 receptor is diluted into Blocking Buffer (*see* **step 10**). Our experience shows that a mix of primary antibody/antiserum plus blocking buffer further reduces background effects in the autoradiography. If the primary antibody is not available, then refer to previously published procedures for preparing primary antibody ***(5,6)***. Our principal consideration for selecting an immunogenic sequence was for developing antisera to be used in Indirect Immunofluorescence microscopy. Thus, the immunogenic sequence was selected from the amino and carboxyl termini of the inferred amino acid sequence based on cDNA information of the MC5-R. Several experimental runs should be performed with different dilutions of antisera to determine the optimal concentration for producing clean immunoblots. A good starting point for immunoblots is an antisera dilution of 1:1000, which may be expanded up to 1:10,000 depending on receptor expression levels and the extent of background interferences.
8. Secondary Antibody: Goat antirabbit IgG conjugated with horseradish peroxidase is diluted according to vendor's directions; this is typically 1:10,000. We generally use a donkey antirabbit IgG antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, NA 934), and detect bound antibody using a reagent kit containing substrates for the horseradish peroxidase activity (Amersham Pharmacia Biotech ECL™, RPN 2006).
9. High Ionic Strength Wash Buffer: 2.7 g NaCl, 0.8 g Trizma®-HCl is dissolved into 90 mL distilled water, and the pH is adjusted to 7.4. Next, 0.1 mL of Nonidet-40 and 0.05 g of sodium deoxycholate is added and the final volume is adjusted to 100 mL with distilled water. The final buffer contains 500 m*M* NaCl, 0.1 % NP40, 0.05% deoxycholate, and 50 m*M* Tris-HCl pH 7.4.
10. Low Ionic Strength Wash Buffer: 0.8 g Trizma®-HCl is dissolved into 90 mL distilled water then 0.1 mL of NP40 and 0.05 g sodium deoxycholate is added. The final buffer contains 50 m*M* Tris-HCl, 0.1% NP40, and 0.05% deoxycholate.

2.2. Equipment

1. Dissection equipment, 100-mm Petri dishes, ice, and ice chest for performing temperature-controlled procedures.
2. Glass dounce homogenizer (10 mL size for small organ preparations).
3. Electrophoresis and transblot equipment.
4. Rocking platform.
5. Syringe (12 mL) and Luer-lock 0.22-μm filters (Millex GV filter unit, cat. SLGVR25LS, Millipore Corp.).
6. Microfuge (capable of generating >10,000*g*).
7. 1.5 mL microfuge tubes.
8. Graduated cylinders (1 L, 100 mL).
9. Heating stirplate and stirbars.
10. Chambers for handling gels and membranes, acrylic storage chamber with lids.
11. Plastic transparency film (3M Corp, Visual Systems Division, Austin TX, cat. PP250).
12. Filter paper.
13. Autoradiography cassettes and film (Hyperfilm™ MP, Amersham Pharmacia Biotech, RPN168K).
14. Timer.

3. Methods

3.1. Tissue Solubilization

1. Carefully dissect the tissue to be analyzed and remove all traces of adipose tissue.
2. Place approx 0.1–1.0 g of tissue into a 100-mm Petri dish setting on top of crushed ice. The tissue is then diced into small cubes (approx 2 mm) using two razor blades sliding in opposite directions to provide a scissoring action.
3. The tissue cubes are placed into a dounce homogenizer (10 mL size) plus 5 mL solubilization buffer (*see* **Subheading 2.1.**). The pestle is then plunged 18–20 times to break up tissue and cells to form a detergent extract. A typical lachrymal gland from an adult rat weighs 150–200 mg wet weight, which provides plenty of material for subsequent immunoblots and immunoprecipitation procedures.
4. The extract is then placed on a rocking platform and mixed for 30 min. Always keep the extract at 4°C by performing the mixing on ice or placing the rocking platform in a cold room.
5. The mixed extract is then clarified to remove large particulate matter by centrifugation at >12,000*g* for 20 min followed by filtration through a 0.22-μm syringe filter.
6. The supernate is quantitated for protein content, diluted to a final concentration of 1–2 mg/mL, and stored at −80°C until needed for electrophoresis (*see* **Note 3**).

Careful consideration should be given to the selection of detergents used for protein solubilization. The potential size determination of an integral protein by SDS-PAGE may differ based on the initial steps used for isolation. Several species of the same protein may be present but differ as to the extent of post-translational modification or association with other proteins or reside in different

membrane lipid-microdomains. The proper detergent selection could dramatically change the apparent results by favoring different forms of the same protein. **Fig. 1** shows a Western analysis of lachrymal tissue detergent homogenates for the expression of MC5 receptor protein. The molecular weight of the recovered MC5 receptor from lachrymal tissue is altered between 68,000–45,000 solely based on the detergent used to make the detergent extract.

3.2. Protein Identification by Immunoblotting

3.2.1. SDS-PAGE

Prepare the SDS-PAGE setup according to the specific protocols provided by the manufacturer. For additional information, *see* **ref *4***. In general, prepare a discontinuous gel for electrophoresis consisting of a 4% stacker gel and an 8% resolving gel. Be sure to note the amount of *bis*-acrylamide cross-linker present in the master mix. The extent of cross-linking will alter the relative migration patterns and resolution of bands of individual protein species in a crude homogenate when compared to the patterns for the standard molecular weight references. **Fig. 2** shows two gels where the master mix for the resolving gels contained different amount of the bis-acrylamide. Note that the higher degree of cross-linking (e.g., 19:1) caused the larger species of the MC5 receptor to run at an apparently higher molecular weight. In addition, the band at 68,000 seen on the 37.5:1 gel appears as four bands in the 19:1 at 58,000, 62,000, 65,000, and 80,000. Finally, we typically cast the resolving gel approx 24 h prior to making the electrophoresis run to ensure complete polymerization; alternatively, we will run the electrophoresis gel for 15 min without sample loaded to allow monomeric acrylamide to migrate out of the gel.

3.2.2. Sample Preparation

1. Mix 2–10 μg of total protein into SDS-PAGE sample buffer. As little as 2.5 μg of solubilized spleen or lachrymal tissue is sufficient for specific detection of the MC5 receptor by Western analysis using chemiluminescence detection. Increasing the amount of detergent extract does not necessarily produce a stronger specific band. The increased sensitivity of detection provided by the chemiluminescence also detects nonspecific binding. We observed abundant proteins could artificially cause band detection in the autoradiographs ***(2)***. The artifactual bands are easy to discern because the competitive peptide will not block detection, but preimmune sera will detect these same bands. Thus several protein concentrations should be tested to detect antibody binding resulting from low abundant proteins.
2. Boil the sample for 2–5 min in a sealed microfuge tube; chill the tube and briefly centrifuge the sample to collect at the bottom.
3. The sample is then transferred to the appropriate well in the stacker gel.

4. Run the electrophoresis at a constant 100 V for 75 min when using the Bio-Rad® Mini-gel apparatus. The determinations of protein size are always approximate by electrophoresis and the protein standards can vary in performance. A good recommendation is to screen different brands for a product that gives standard sizes in a useful range and color coded for different sizes.

3.2.3. Protein Blotting

1. Electrophoretic transfer of resolved proteins from the gel is performed by standard techniques, *see* **ref. 7** for general details. We use a nitrocellulose paper with a pore size of 45 μm (Schleicher & Schuell 62660 or Amersham Hybond-ECL RPN 2020-D). The nitrocellulose paper is carefully cut into rectangular pieces and wetted by placing into fresh transfer buffer.
2. At the same time, the electrophoresis gel from **Subheading 3.2.2.** is removed from the apparatus and placed into a separate container and washed with transfer buffer. The washing step is critical to remove excess SDS and other solutes. We recommend soaking the gel for at least 2 min, aspirating the buffer and repeating the wash a second time.
3. The washed gel is then placed into the same container with the nitrocellulose, membrane and sandwiched between two rectangular sheets of filter paper. The gel, nitrocellulose and filter paper sandwich is mounted between two porous pads in the cassette and placed into the transfer chamber. Orient the sandwich so that the nitrocellulose paper is on the anode side and the gel on the cathode side (remember that SDS-bound proteins will "run to red").
4. The transblot is run at 100 V for 90 min. Several transfers may be needed to optimize run times so all important proteins species are transferred to the paper. Longer run times are required for larger proteins.

3.2.4. Antibody Detection and Autoradiography

1. Immediately following the protein blotting, the membrane is separated from the sandwich and readied for antibody probing.
2. The protein-depleted gel is placed into a solution of Coomassie blue (Bio-Rad Laboratories, cat. 161–0436) to check for the transfer efficiency.
3. The gel is then washed three times with a solution of methanol:acetic acid:water (5:1:5). The gel should be clear of all proteins following the destaining step.
4. The membrane containing transferred proteins is first washed with 50 mL of PBST by allowing the membrane to soak for 30 before aspirating the PBST. This step is repeated three times.
5. The washed membrane is then placed in blocking buffer at 4°C overnight.
6. The blocked membrane is next washed to remove excess milk by aspirating the blocking buffer; replacing with PBST, soaking for 30; and the PBST wash is repeated three times.
7. The primary antibody specific for the MC5 receptor is diluted into blocking buffer; we typically dilute 1:1000 by mixing 20 μL antisera with 20 mL solution. The membrane is incubated with the primary antibody for 2 h at room temperature.

8. The primary antibody is then removed by aspiration and the bound membrane is again washed. The first two washes are soaked for 30s in PBST; the third wash is for 15 min in PBST followed, by two more washes with 5-min soaking intervals. The third wash has been found to be very crucial toward giving clean responses and should last at least 15 min.
9. Finally, the secondary antibody (an antirabbit antibody conjugated with horseradish peroxidase) should also be diluted with blocking buffer; we typically dilute 1:10,000 by mixing 2 μL conjugated antibody with 20 mL of solution. The bound membrane is then incubated with the secondary antibody for 1 h at room temperature. The membrane must be washed using the same sequence listed for the primary antibody.
10. Finally, the membrane is placed on top of a sheet of plastic (transparency plastic used for overhead projectors) and the chemiluminescence's reagent mix is added. A second piece of plastic sheeting is placed on top and the air bubbles are carefully removed by a squeegee action. The membrane sandwich is then placed into an autoradiography cassette with film. We typically expose the film between 2–30s, and with low abundant expression of the MC5 receptor the exposure may be extended to 2 min. Our experience is that increased exposure time lead to the detection of nonspecific bands and increased background noise, thus, longer exposure times are not likely to yield improved detection.

3.3. Isolation of Receptor Protein by Immunoprecipitation Using Protein A-Agarose

3.3.1. Sample Preparation

1. A sample of clarified detergent extract (*see* **Subheading 3.1.**) is diluted in solubilization buffer to 1 mL in a 1.5-mL microcentrifuge tube. The final protein concentration should be in the range of 0.5–0.75 mg/mL.
2. The diluted sample is first precleared to remove proteins that nonspecifically bind the Protein A-agarose (Boehringer Mannheim, cat. 1719408) independent of antibody.
3. Protein A-agarose suspension (50 μL) is added to the diluted sample and mixed on a rocking platform for 3 h at 4°C.
4. The Protein A-agarose is then removed by centrifugation (12,000*g*, for 30 s).
5. The precleared supernate is then recovered by gentle pipeting and placed into a second microcentrifuge tube.

3.3.2. Antibody Capture of MC5 Receptor

1. Antireceptor antibody is then added to the precleared sample; in our hands, the antisera to MC5-R is added to a 1:500 dilution (2 μL antisera to the 1 μL of precleared sample).
2. The sample is again mixed on a rocking platform for 1 h at 4°C.
3. Antibody bound material is selectively removed by adding fresh Protein A-agarose (50 μL to the 1 mL sample) and mixing on a rocking platform for 24 h at 4°C.
4. The Protein A-agarose containing antibody captured receptor is then removed by centrifugation (12,000*g* for 30 s at 4°C).

5. The supernate is now removed by aspiration leaving capture MC5-R bound to the pelleted Protein A-agarose.

3.3.3. Washing Captured MC5-R

1. The Protein A-agarose pellet is now washed to remove nonspecifically bound proteins. The Protein A-agarose is first resuspended into 1 mL of tissue solubilization buffer (*see* **Subheading 2.1.**) and gently mixed on a rocking platform for 20 min at 4°C.
2. The suspension is pelleted by centrifugation (12,000*g* for 30 s) and the supernate removed by aspiration.
3. This procedure is then repeated once more using solubilization buffer, and two times using the high salt buffer, followed by a single wash step using the low salt buffer.

3.3.4. Western Blot Analysis of Captured MC5-R

The washed Protein A-agarose coupled with antibody and specific bound protein is then analyzed by SDS-PAGE/Western. The sample can be placed into gel loading buffer, the 2-mercaptoethanol reducing reagent and high temperature will release the agarose bound antibody and receptor proteins. This procedure provides for very good recovery of the receptor protein, however, dissociated antibody will also be recovered. The sample can now be analyzed by SDS-PAGE/Western. The immunoglobulin light (approx 22,000) chain will run close to the dye front, and the immunoglobulin heavy chain for IgG will run around 55,000. An additional clean-up step can include size-exclusion chromatography to separate the heavy chain immunoglobulin subunit from the receptor.

4. Notes

1. The stock PMSF should be stored <–20°C, and has a short half-life once diluted into the tissue solubilization buffer.
2. We screened several different grocery store brands of milk to identify a brand giving the lowest background artifacts. We have also used 1% bovine serum albumin and Sea Block® (Pierce Chemical, Rockford IL, cat. 37527) but the best results appear to come from using inexpensive instant nonfat dry milk. Dry milk should not be used if the primary antibody is directed to a phospho-amino acid; the casein in milk is highly phosphorylated and will cause excessive background noise.
3. Clarification is used to preserve chromatography resins. Because partially solubilized receptor may be lost at this stage, then the extract may be used for direct application to electrophoresis. In this latter case, the stronger SDS detergent in the electrophoresis sample buffer is likely to increase solubilization and can be used to save time during the initial stages of working up an individualized protocol. We generally use a detergent insensitive protein assay (NI Protein Assay, Geno Technology, Inc., St. Louis, MO).

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14

Purification of Mammalian Serine Palmitoyltransferase, a Hetero-Subunit Enzyme for Sphingolipid Biosynthesis, by Affinity-Peptide Chromatography

Kentaro Hanada and Masahiro Nishijima

1. Introduction

Serine palmitoyltransferase [SPT (EC 2.3.1.50)] is the enzyme responsible for the condensation of L-serine with palmitoyl CoA to produce 3-ketodihydrosphingosine (KDS), the reaction of the first step in sphingolipid biosynthesis ***(1,2)***. SPT is a membrane-bound enzyme of the endoplasmic reticulum, protruding its catalytic site to the cytoplasm ***(3,4)***, and probably plays an important role in the regulation of the levels of total sphingolipids in cells ***(5–7)***. Genetic and biochemical studies have revealed that at least two different genes, *LCB1* and *LCB2*, are required for expression of SPT activity ***(8–12)***, and that both the LCB1 and LCB2 proteins are subunits of the SPT enzyme ***(13,14)***.

Characterization of purified SPT is necessary for better understanding this key enzyme of sphingolipid biosynthesis. However, the nature of the SPT enzyme is in itself an obstacle to its purification. First, only a few types of detergents are suitable for solubilization of this membrane-bound enzyme without inactivation, narrowing conventional chromatographic methods applicable to the purification of SPT. Second, no affinity ligands useful for purification of active SPT are so far known. Third, because SPT consists of different subunits, overproduction of one subunit type alone does not facilitate purification of this enzyme complex. Similar problems cause the general difficulty in purification of various types of multimeric membrane proteins, even when genes encoding their subunits have been identified. Thus, development of rational purification

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strategy applicable to various types of membrane proteins has been desired for investigation of membrane proteins in purified forms.

If an affinity-peptide-tagged subunit is functionally integrated to a multimeric protein complex in cells, the multimeric protein complex may be purified in an active form by affinity chromatography for the peptide tag. We showed that a FLAG- and hexahistidine (His_6)-tagged version of the hamster LCB1 protein functionally substitutes for the wild-type LCB1 protein in Chinese hamster ovary (CHO) cells ***(14)***. Then, we purified active SPT from the CHO cells expressing the doubly tagged LCB1 protein to homogeneity by affinity-peptide chromatography ***(14)***. In this chapter, the method for the purification of the affinity-peptide tagged SPT complex is described.

2. Materials

Water purified with the Milli-Q system (Millipore, Bedford, MA) is used.

2.1. Construction of pSV-FHcLCB1

1. Hamster *LCB1* cDNA (c*LCB1*) cloned into pSPORT1 vector ***(12)***.
2. Restriction enzymes (*Bsr* GI, *Nco* I *Sal* I, *Not* I, and *Pst* I) are from New England BioLabs, Inc. (Beverly, MA).
3. Synthesized oligonucleotides (5'-TCGACCATGGCGCATCACCATCACCATCA-3'; 5'-CATGTGATGGTGATGGTGATGCGCCATGG-3'; 5'-TTTAGTCGACCATGGACTACAAGGACGACGATGACAAACATCACCATCACCATC-3'; and 5'-CCAGGCGCTCTTCCAAATCC-3') are from Sawady Technology Inc. (Tokyo, Japan).
4. pBluescript (Stratagene, La Jolla, CA).
5. pSV-OKneo, a mammalian expression vector ***(12)***.
6. Automated DNA sequencer (ABI PRISM™ 310, Applied Biosystems).

2.2. Cell Culture and Preparation of Membranes

1. The LY-B strain, a CHO cell mutant strain defective in SPT owing to the lack of expression of the LCB1 protein, is from the stock of our laboratory ***(13)***.
2. Lipofectamine PLUS™ reagent (Invitrogen).
3. G418 (Sigma).
4. ES medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 2 m*M* L-glutamine, $NaHCO_3$ (1 g/L), 10 m*M* HEPES-NaOH (pH 7.4) and 5% (v/v) fetal calf serum.
5. Phosphate-buffered saline (PBS).
6. 10 m*M* sucrose containing 1 m*M* ethylenediaminetetraacetic adic (EDTA).
7. 40-mL Dounce tissue grinder (pestle type A, Wheaton, Millville, NJ).
8. 2 *M* sucrose.
9. 10 m*M* HEPES-NaOH buffer (pH 7.5) containing 250 m*M* sucrose.

2.3. Purification of SPT by Affinity-Peptide Chromatography

1. Sucrose monolaurate (Dojindo Laboratories, Kumamoto, Japan).
2. Phosphatidylcholine (PC): Egg PC dissolved in chloroform is from Avanti Polar Lipids, Inc.
3. FLAG peptide (Sigma).
4. Anti-FLAG M2 affinity gel (Sigma).
5. Ni-nitrilotriacetic acid (Ni-NTA) agarose is from Qiagen GmbH (Hilden, Germany).
6. Concentrator: Ultrafree®-4 (Millipore).
7. Probe-type sonicator.
8. Column: 5-mL polypropylene column (Pierce).

2.3.1. Stock Solutions for Affinity-Peptide Chromatography

1. 20% (w/v) sucrose monolaurate (*see* **Note 1**), stored at −20°C.
2. PC (5 mg/mL) (*see* **Note 2**): 25 mg of PC dissolved in chloroform is put in a thick glass tube. After complete removal of the organic solvent under a nitrogen stream in a ventilated chemical hood, dried PC is sonicated in 5 mL of water for ~3 min with a probe-type sonicator. The stock of sonicated PC can be stored at −80°C for 2–3 mo. The frozen stock is thawed and sonicated briefly at room temperature prior to use.
3. FLAG peptide (5 mg/mL), stored at −20°C.
4. 5 *M* NaCl is autoclaved, and stored at room temperature.
5. 100 m*M* glycine-HCl buffer (pH 3.5), stored at 4°C.
6. 50 m*M* Tris-HCl buffer (pH 7.4) containing 150 m*M* NaCl, stored at 4°C.
7. 200 m*M* sodium phosphate buffer (pH 8.0), stored at 4°C.
8. 1 *M* imidazole (neutralized to pH 8.0 with 1 *N* HCl), stored at 4°C.
9. 2 *M* sucrose, stored at 4°C.
10. 500 m*M* HEPES-NaOH buffer (pH 7.5), stored at −20°C.

2.3.2. Buffers for Affinity-Peptide Chromatography

The following buffers are freshly prepared on the day of use from the aforementioned stock solutions, and chilled on ice.

1. 125 m*M* sodium phosphate buffer (pH 8.0) containing 188 m*M* NaCl.
2. FW1: 100 m*M* sodium phosphate buffer (pH 8.0) containing 50 m*M* NaCl.
3. FW2: 100 m*M* sodium phosphate buffer (pH 8.0) containing 50 m*M* NaCl, 0.1% sucrose monolaurate, and 0.1 mg/mL PC.
4. FE: 100 m*M* sodium phosphate buffer (pH 8.0) containing 50 m*M* NaCl, 0.1% sucrose monolaurate, egg PC (0.1 mg/mL), and FLAG peptide (120 μg/mL).
5. NW1: 100 m*M* sodium phosphate buffer (pH 8.0) containing 100 m*M* NaCl, 10 m*M* imidazole, and 0.1% sucrose monolaurate.
6. NW2: 100 m*M* sodium phosphate buffer (pH 8.0) containing 100 m*M* NaCl, 10 m*M* imidazole, 0.1% sucrose monolaurate, and egg PC (0.1 mg/mL).
7. NE: 100 m*M* sodium phosphate buffer (pH 8.0) containing 100 m*M* NaCl, 250 m*M* imidazole, 0.1% sucrose monolaurate, and egg PC (0.1 mg/mL).

8. HSS: 10 m*M* HEPES-NaOH buffer (pH 7.5) containing 250 m*M* sucrose, and 0.1% sucrose monolaurate.

2.4. Assay of SPT Activity

1. L-[^{3}H(G)]serine (20 Ci/mmol) is from American Radiolabeled Chemicals (St. Louis, MO).
2. Nonradioactive L-serine (Sigma).
3. Standard SPT reaction buffer: 50 m*M* HEPES-NaOH buffer (pH 7.5) containing 5 m*M* EDTA, 5 m*M* dithiothreitol (DTT), 50 μ*M* pyridoxal phosphate, 25 μ*M* palmitoyl CoA, and 0.1 m*M* L-[^{3}H(G)]serine (50 mCi/mmol).

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Silver Stain, and Western Blot

1. SDS-sample buffer: 100 m*M* Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, and 13 μg/mL bromphenol blue.
2. 10–20% (w/v) gradient gel (PAGEL®, Atto Inc., Tokyo, Japan).
3. 7.5% (w/v) gel supplemented with 3 *M* urea.
4. Molecular mass standards (Bio-Rad).
5. Silver staining kit (Wako, Osaka, Japan).
6. polyvinylidene difluoride membrane (Bio-Rad).
7. PBS containing 0.1% Tween-20 (PBS-T).
8. PBS-T with 10% (w/v) skim milk.
9. PBS-T with 1% (w/v) skim milk.
10. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad).
11. Enhanced chemiluminescence kit (Amersham Pharmacia Biotech).
12. Antihamster LCB1 and antihamster LCB2 antibodies ***(13,14)***.

2.6. Protein Determination

1. BCA protein assay kit (Pierce).

3. Methods

3.1. Construction of pSV-FHcLCB1, a Recombinant Plasmid Encoding a FLAG- and His_6-Tagged LCB1 Protein

We first constructed pSV-HTcLCB1 encoding *His*$_6$-tagged version of the cLCB1 protein ***(12)***, and, then, reconstructed it to pSV-FHcLCB1 encoding the doubly tagged version of the cLCB1 protein (14) (*see* **Fig. 1;** *see* **Note 4**).

1. The hamster *LCB1* cDNA (c*LCB1*) cloned into pSPORT1 vector is digested with *Bsr* GI and is self-ligated to delete a *Nco* I site of the 3'-untranslated region.
2. The resultant plasmid is digested with *Nco* I and *Sal* I and, then, is ligated with annealed oligonucleotides (5'-TCGACCATGGCGCATCACCATCACCATCA-3' and 5'-CATGTGATGGTGATGGTGATGCGCCATGG-3') encoding a *His*$_6$-tag sequence.

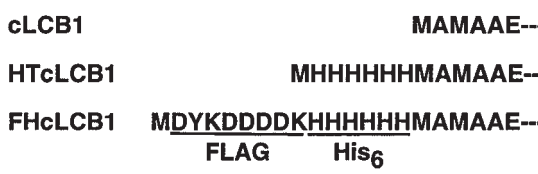

Fig. 1. Affinity-peptide tagged versions of the hamster LCB1 protein. The amino terminal sequences of the wild-type hamster LCB1 (cLCB1), *His*$_6$-tagged LCB1 (HTcLCB1), and FLAG/*His*$_6$-tagged LCB1 (FHcLCB1) are shown.

3. The *Sal* I-*Not* I fragment of the *His*$_6$-tagged c*LCB1* is transferred from pSPORT1 to pSV-OK*neo*, a mammalian expression vector, and the resultant construct is designated pSV-HTcLCB1 ***(12)***.
4. For introduction of a FLAG sequence prior to the His$_6$ sequence, PCR is performed with pSV-HTcLCB1 as the template, a synthetic oligonucleotide encoding the FLAG sequence (5'-TTTAGTCGACCATGGACTACAAGGACGACGATGACAAACATCACCATCACCATC-3') as the forward primer, and an oligonucleotide (5'-CCAGGCGCTCTTCCAAATCC-3') as the reverse primer.
5. Approx 400-bp *Sal* I-*Pst* I fragment of the PCR product replaces the corresponding region of HTcLCB1 cloned into pBluescript to make the FHcLCB1 sequence.
6. The *Sal* I-*Not* I fragment of the FHcLCB1 sequence is transferred from pBluescript to pSV-OK*neo*, and the resultant construct is designated pSV-FHcLCB1 ***(14)***.
7. The nucleotide sequence of the construct is verified by using an automated DNA sequencer.

3.2. Cell Culture and Preparation of Membranes

1. LY-B cells are transfected with pSV-FHcLCB1 by using the Lipofectamine PLUS™ reagent.
2. After selection with G418 (400 μg/mL), several colonies resistant to the drug are purified, propagated, and assayed for SPT activity.
3. Among transfected LY-B clones showing recovery of SPT activity, one clone designated as LY-B/FHcLCB1 strain is chosen for purification of the SPT enzyme complex ***(14)***.
4. LY-B/FHcLCB1 cells are cultivated in spinner bottles containing 1 L of ES medium supplemented with 2 m*M* L-glutamine, $NaHCO_3$ (1 g/L), 10 m*M* HEPES-NaOH (pH 7.4) and 5% (v/v) fetal calf serum at 37°C. Thereafter, all manipulations are done at 4°C or on ice.
5. Cells from 5-L cultures are harvested by centrifugation (600*g*, 5 min), and washed with 250 mL of PBS.
6. The washed cells are suspended in 30 mL of 10 m*M* sucrose containing 1 mM EDTA, and homogenized with a 40-mL Dounce tissue grinder by 70 strokes.
7. The homogenate is centrifuged (1000*g*, 15 min) to remove cell debris and nuclei.
8. After addition of 2 *M* sucrose to the supernatant to achieve a final concentration of 0.25 *M*, the resultant sample is centrifuged (105^5g, 1 h).

9. The precipitate, as intact membranes, is suspended in 10 m*M* HEPES-NaOH buffer (pH 7.5) containing 250 m*M* sucrose at approx 10 mg protein/mL with a syringe equipped with a 24-gage needle, and stored at −80°C until use.

3.3. Purification of SPT by Affinity-Peptide Chromatography

All manipulations are done at 4°C or on ice.

3.3.1. Preequilibration of Resins

1. Anti-FLAG M2 affinity gel is prewashed by 5 mL of 100 m*M* glycine-HCl buffer (pH 3.5), and neutralized by washing three times with 5 mL of 50 m*M* Tris-HCl buffer (pH 7.4) containing 150 m*M* NaCl, according to the manual of the manufacturer. Then, the gel (0.2 mL bed volume per microfuge tube) is washed twice with 1 mL of FW1, and subsequently washed twice with 1 mL of FW2 prior to use (*see* **Note 3**).
2. Ni-NTA agarose (0.1 mL bed volume per microfuge tube) is washed twice with 0.5 mL of NW1, and subsequently twice with 1 mL of NW2 prior to use (*see* **Note 3**).

3.3.2. Solubilization of Membranes

1. The membrane suspension is centrifuged (10^5g, 30 min).
2. The precipitated membranes (approx 20 mg protein) are suspended in 4 mL of 125 m*M* sodium phosphate buffer (pH 8.0) containing 188 m*M* NaCl.
3. 0.5 mL of 5 mg/mL phosphatidylcholine and 0.5 mL of 20% (w/v) sucrose monolaurate are added to the membrane suspension, mixed, incubated for 10 min, and centrifuged (10^5g, 30 min).
4. The supernatant fluid is recovered as the solubilized membrane fraction. Solubilization efficiency of SPT activity is almost 100% (*See* **Table 1**).

3.3.3. FLAG-Affinity Chromatography

1. The solubilized membrane fraction is incubated with 0.5 mL of anti-FLAG M2 affinity gel, which was equilibrated with buffer FW2, for 90 min with rotary shaking.
2. The resin is precipitated by centrifugation (10^4g, 20 s), suspended in 0.5 mL of buffer FW2, and transferred to a column.
3. The resin in the column is washed with 15 mL of buffer FW2 and then eluted with 4.5 mL of buffer FE (*see also* **Note 5**). The elution is collected in a 15-mL polypropylene tube.

3.3.4. His_6-Affinity Chromatography (see ***Note 5***)

1. 1 *M* imidazole is added to the elution fraction of FLAG-affinity chromatography to achieve a final concentration of 10 m*M*.
2. This sample is incubated with 0.5 mL of Ni-NTA agarose, which was equilibrated with buffer NW2, for 1 h with rotary shaking (*see* **Note 6**).

Table 1
Purification of the SPT Enzyme Having Double Affinity-Peptide Tags

Fraction	Protein (μg/fraction)	SPT activity per fraction (pmol/min)	SPT specific activity (nmol/mg protein/min)	Enrichment (Fold)
Detergent-treated membranes[a]	16,700	885	0.053	1.0
Solubilized membrane[b]	14,200	895	0.063	1.2
Elution of anti-FLAG M2 affinity gel	4.8[c]	264	55.1	1050
Elution of Ni-NTA agarose	1.2[c]	163	136	2580

[a]Membranes incubated with 2% (w/v) sucrose monolaurate for 10 min.

[b]Supernatant fluid after centrifugation ($10^5 g$, 30 min) of the detergent-treated membranes.

[c]Protein concentrations were estimated by densitometric comparison of silver-stained proteins of the fraction with stained calibration bands of bovine serum albumin of known concentrations in SDS-PAGE.

3. The resin is precipitated by centrifugation (1000*g*, 1 min).
4. The precipitated resin is suspended in 10 mL of buffer NW2 and precipitated for washing
5. This washing step is repeated twice more.
6. The precipitated resin is suspended with 5 mL of buffer NE, and incubated for 10 min (*see* **Note 6**).
7. The resin is precipitated by centrifugation (1,000*g*, 1 min), and the supernatant fraction is collected as the elution fraction of His_6-affinity chromatography.

FLAG-affinity chromatography is very effective, but not enough to purify SPT to homogeneity (*see* **Table 1** and **Fig. 2**). However, the doubly tagged SPT complex is purified to homogeneity by the combination of two types of affinity-peptide chromatography shown above (*see* **Table 1** and **Fig. 2**) (*see also* **Notes 7** and **8**).

3.3.5. Storage of Purified SPT

1. For removal of imidazole, the elution fraction of His_6-affinity chromatography is diluted five-fold with buffer HSS, and concentrated to 1–2 mL by ultrafiltration with Ultrafree®-4.
2. These dilution and concentration steps are repeated twice.
3. The purified SPT fraction is divided and stored at −80°C until use (*see* **Note 9**).

3.4. Assay of SPT Activity

1. The enzyme source is incubated in 200 μL of the standard SPT reaction buffer at 37°C for 10 min.
2. After stopping the reaction, lipids are extracted. The radioactivity of the KDS that formed is measured as aforementioned ***(15)***. The radioactivity extracted from enzyme-minus controls is regarded as a background control.

3.5. SDS-PAGE, Silver Stain, and Western Blot

SDS-PAGE is carried out by a modification of the method of Laemmli ***(16)***:

1. Samples for SDS-PAGE are incubated in a SDS-sample buffer at 37°C for 15 min.
2. Two types of polyacrylamide gel are used: a ready made 10–20% (w/v) gradient gel and a hand made 7.5% (w/v) gel supplemented with 3 *M* urea.
3. Proteins separated on gel are stained with a silver staining kit.
4. For Western blot analysis, proteins separated by SDS-PAGE are transferred to a polyvinylidene difluoride membrane.
5. The blot membrane is incubated in 10 mL of PBS-T containing 10% skim milk for 1 h with gentle shaking.
6. The membrane is incubated in 5 mL of PBS-T containing 1% skim milk and the primary antibody (0.5 μg/mL of antihamster LCB1 antibody or antihamster LCB2 antibody) for 1 h.
7. The membrane is washed three times with 10 mL of PBS-T for 15 min.

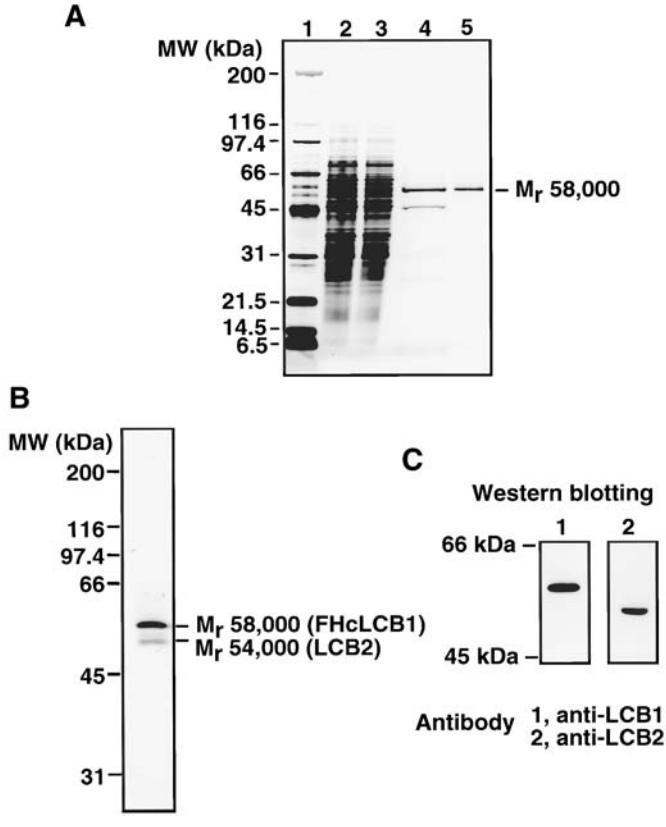

Fig. 2. SDS-PAGE of the different steps of the SPT purification. (**A**) Silver-stained patterns with 10–20% (w/v) gradient acrylamide gel. Lane 1, molecular mass standards (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.5 kDa; aprotinin, 6.5 kDa); lane 2, solubilized membrane fraction; lanes 3 and 4, flowthrough fraction and elution fraction, respectively, of anti-FLAG M2 affinity chromatography; lane 5, elution fraction of Ni-NTA agarose chromatography. **(B)** Elution fraction of Ni-NTA agarose chromatography separated by SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 *M* urea, and stained by silver staining. **(C)** Western blotting of purified SPT. The elution fraction of Ni-NTA agarose chromatography was separated by SDS-PAGE with 7.5% (w/v) acrylamide gel containing 3 *M* urea, and analyzed by Western blotting with an antihamster LCB1 antibody (lane 1) or antihamster LCB2 antibody (lane 2).

8. The membrane is incubated in 5 mL of PBS-T containing 1% skim milk and a horseradish peroxidase-conjugated goat antirabbit IgG (1:2500 dilution).
9. The membrane is washed three times with 10 ml of PBS-T for 15 min.
10. The membrane is processed with an enhanced chemiluminescence kit, according to the manual of the manufacturer.

3.6. Protein Determination

Protein concentrations is determined with the Pierce BCA protein assay kit using bovine serum albumin as the standard, according to the manual of the manufacturer.

4. Notes

1. After examining various types of detergents (e.g., Triton X-100, octylglucoside, cholic acid, and CHAPS), for efficient and stable solubilization of active SPT, we found that sucrose monolaurate is the best for this purpose.
2. SPT can be solubilized efficiently from membranes by sucrose monolaurate even in the absence of PC. However, elimination of phospholipids during the purification process destabilized the activity of solubilized SPT. The loss of the activity was significantly prevented by the presence of PC in buffers throughout the purification.
3. For wash of resins in microfuge tubes, resins are precipitated by centrifugation (10,000 rpm, 20 s), and, after careful removal of the supernatant, suspended with the indicated buffers.
4. We initially attempted to purify the SPT complex from LY-B cells expressing the His_6-tagged cLCB1 protein by His_6-affinity chromatography alone, but found that this method was quite insufficient for the purification to homogeneity. Thus, we decided to introduce another affinity-peptide sequence, FLAG, to the amino terminus of the His_6-tagged cLCB1 protein. If one wants to construct a plasmid for expression of a similar doubly tagged protein, the FLAG- and His_6-sequence codons may be simultaneously introduced to the target protein cDNA by a single PCR with an appropriate set of primers.
5. Affinity-peptide tagged proteins bound to these affinity resins can be eluted under mild conditions by addition of appropriate competitors (FLAG peptide for anti-FLAG M2 affinity gel, and imidazole for Ni-NTA agarose).
6. When solubilized membrane fraction was incubated with Ni-NTA agarose in 50 m*M* HEPES-NaOH buffer (pH 7.5) containing 250 m*M* NaCl, most of proteins were adsorbed nonspecifically to the resin (our unpublished results). The nonspecific binding was greatly improved by using 100 m*M* sodium phosphate buffer (pH 8.0) containing 100 m*M* NaCl and 10 m*M* imidazole as the basal buffer in the binding step (our unpublished results). Probably, Ni-NTA agarose has a weak affinity to phospholipids, due to interaction between Ni^{2+} and the phosphate ester group of phospholipids, thereby nonspecifically trapping membrane proteins embedded in phospholipid/detergent mixed micelles. Such nonspecific binding via a relatively weak interaction

between Ni-NTA and phospholipid/detergent mixed micelles seems to be abrogated by the presence of 100 m*M* inorganic phosphate and 10 m*M* imidazole. The His_6-tagged SPT complex bound to Ni-NTA agarose in the presence of 10 m*M* imidazole can be eluted by the incubation with 250 m*M* imidazole (*see* **Table 1**).

7. When SDS-PAGE was performed with the ordinary polyacrylamide gel, the elution fraction of His_6-affinity displayed only one visible band on silver stain analysis of SDS-PAGE (*see* **Fig. 2A**). However, Western blot analysis with antihamster LCB1 and LCB2 antibodies revealed that the FHcLCB1 protein coincidentally overlapped the LCB2 protein in the SDS-PAGE gel. SDS-PAGE with urea-containing gel separated the FHcLCB1 and LCB2 proteins, allowing us to display two bands (M_r 58,000 and M_r 54,000) in the highly purified SPT fraction (*see* **Fig. 2B**). Based on Western blot analysis, the M_r 58,000 protein in the urea-containing SDS-PAGE gel was identified as the FHcLCB1 protein, and the Mr 54,000 protein as the LCB2 protein (*see* **Fig. 2C**). The reason for the M_r value change of the LCB2 protein depending on the presence of urea in SDS-PAGE is unclear.
8. For elucidation of the subunit stoichiometry of the SPT complex, the purified SPT fraction we obtained had a shortcoming. Some FHcLCB1 protein molecules not associated with the LCB2 protein were also accumulated during the affinity-peptide-mediated purification process. Thus, we used another method to determine the subunit ratio in the wild-type SPT complex. In brief, after metabolic labelling of proteins in CHO-K1 cells with a ^{35}S-Met/Cys protein labeling mix, the membranes of the cells were solubilized under conditions where SPT sustained the activity, and subjected to immunoprecipitatation with the anti-LCB2 antibody. Then, the ratio of the radioactivity of the LCB1 subunit to the LCB2 subunit in the immunoprecipate was determined, and, after normalization to the numbers of the deduced *Met* and *Cys* residues, the molecular ratio of these subunits was estimated. This analysis suggested that the SPT enzyme consisted of LCB1 and LCB2 subunits with a molecular ratio of 1:1 ***(14)***
9. The frozen stock of purified SPT retains the activity for at least 2 wk, but prolonged storage causes gradual loss of the activity. Storage at 4°C causes more rapid loss of the activity. Repeat of freeze and thawing inactivates the purified SPT.

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15

Purification of Phosphatidylglycerophosphate Synthase from Cultured Mammalian Cells

Kiyoshi Kawasaki and Masahiro Nishijima

1. Introduction

The biosynthetic pathways for phosphatidylglycerol (PG) and cardiolipin (CL) in mammalian cells have been established by early enzymological studies, as shown in **Fig. 1** (*see* **ref. *1***). Phosphatidylglycerophosphate (PGP) synthase catalyzes the first step in the CL branch of phospholipid biosynthesis with the displacement of the CMP moiety of CDP-diacylglycerol by L-glycerol 3-phosphate to produce PGP ***(2)***. Then PGP is rapidly dephosphorylated to generate PG that is utilized as a substrate along with CDP-diacylglycerol for CL synthesis. Because PGP synthase activity is high in the inner mitochondrial membrane ***(2–4)***, the PGP synthase protein is thought to be abundant in the inner membrane.

It has been shown that PGP synthase is a crucial enzyme for the biosyntheses of PG and CL by the isolation of a temperature-sensitive Chinese hamster ovary (CHO) cell mutant, PGS-S, which is defective in PGP synthase activity ***(5)***. The PGP synthase activity in PGS-S is only 1% of that in wild type CHO-K1 cells, and the biosynthetic rates for and cellular contents of PG and CL are also markedly reduced in the mutant ***(5)***. Previously, we reported the isolation of a CHO *PGS1* cDNA ***(6)*** encoding a putative protein similar in sequence to the yeast *PGS1* gene product, PGP synthase ***(7)***. The expression of CHO *PGS1* cDNA complements the defect in PGP synthase of PGS-S cells, and corrects the reduced PG and CL contents in the mutant ***(6,8)***. These results indicate that CHO *PGS1* encodes PGP synthase. Although the cDNA of PGP synthase have been isolated, the catalytic and regulatory mechanisms, and structure of PGP synthase remain to be elucidated, partly because the complete purification of PGP synthase from a mammal has not been achieved yet. Recently, we reported

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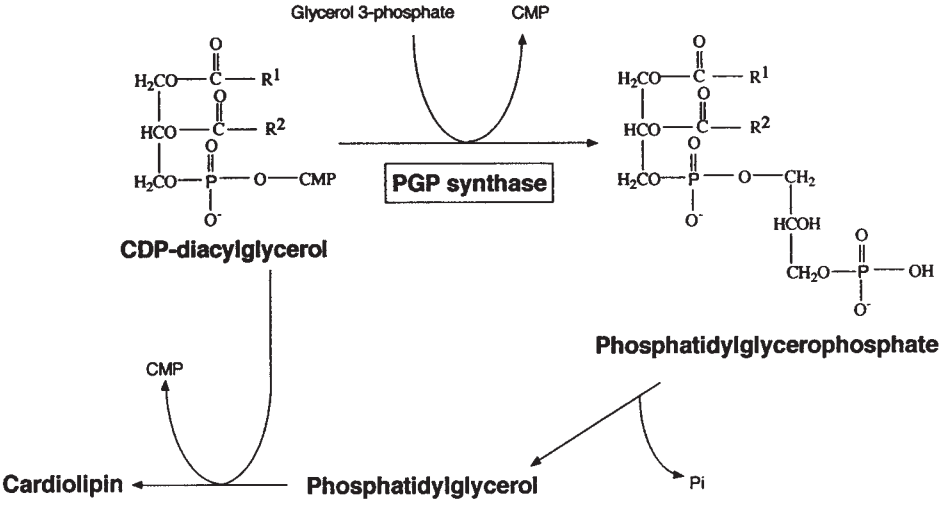

Fig. 1. Biosynthetic pathways for PG and CL in mammalian cells.

the purification of PGP synthase *(9)*. In this chapter, we describe the purification protocol for PGP synthase from wild type CHO-K1 cells.

As shown in **Table 1**, the overall purification of the enzyme from the postnuclear supernatant (P. N. S.) of wild-type CHO-K1 cells was 18,000-fold, with a recovery yield of 0.049% (*see* **Note 1**). The proteins at the different steps of the PGP synthase purification were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The final enzyme preparation gave an apparent single band corresponding to a molecular mass of 60 kDa on silver staining (*see* **Fig. 2**). The enzyme activity was well correlated with the amount of the 60-kDa protein in the final fractions with TSK-Ether (*see* **Fig. 3**). In addition, the enzyme reaction products were analyzed by TLC, and it was confirmed that PGP is the reaction product of the purified enzyme (*see* **Fig. 4**).

2. Materials

All chemicals were of reagent grade or better.

1. Thin-layer chromatography (TLC) Silica Gel 60 plates (Merck).
2. L-[U-^{14}C]glycerol 3-phosphate (ICN Radiochemicals).
3. CDP-diacylglycerol (Serdary Research Laboratory).
4. L-Glycerol 3-phosphate and Triton X-100 (Sigma Ultra, Sigma Aldrich).
5. Chloroform/methanol/0.1 *M* KCl (3:47:48, by vol).
6. Chloroform/methanol (1:2, v/v).
7. 0.5 N oxalic acid.

Table 1
Purification of PGP Synthase From CHO Cells

Purification step	Protein (*mg*)	Total activity (*nmol/min*)	Yield (*%*)	Specific activity (*nmol/min; shmg*)	Purification (*Fold*)
PNS	12,000	7800	100	0.65	1
Purified mitochondria	250	1270	16	5.1	7.8
Solubilized mitochondrial proteins	200	1000	13	5.0	7.7
Blue-Sepharose	5.0	300	3.8	60	92
Mono Q	ND*	26	0.33	ND	ND
Superdex 200	ND	16	0.21	ND	ND
TSK-Ether	0.00032[d]	3.8	0.049	12,000	18,000

*ND: not determined.

[d] Protein concentrations were determined by densitometric comparison of silver-stained proteins in the fractions with stained calibration bands of BSA at known concentrations on SDS-PAGE.

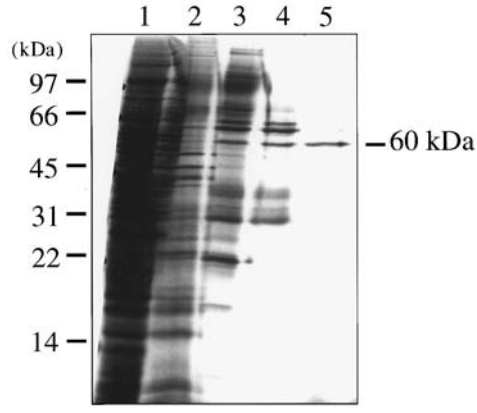

Fig. 2. SDS-PAGE analysis of proteins at different steps of PGP synthase purification. Aliquots of proteins, which contained equal levels of enzyme activity (140 pmol/min), were analyzed by SDS-PAGE on a 12.5% acrylamide gel, and visualized by silver staining. Lane 1, solubilized mitochondrial proteins; lanes 2–5, pooled active fractions from the Blue-Sepharose (lane 2), Mono Q HR 5/5 (lane 3), Hi Load 16/60 Superdex 200 (lane 4), and TSK-Ether (lane 5) columns. The molecular mass of the purified protein (60 kDa) was estimated by comparison with molecular mass standards (phospholylase, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 22 kDa; lysozyme, 14 kDa), the sizes being indicated at the left.

8. Chloroform/methanol/ 35% HCl (87:13:0.2, by vol).
9. BCA protein assay reagent and bovine serum albumin (BSA) protein standard (Pierce).
10. Strain CHO-K1 (American Type Culture Collection).
11. ES medium (Nissui Pharmaceutical Co., Ltd.).
12. Fetal bovine serum (FBS-JRH Bioscience).
13. Blue-Sepharose, Mono Q HR 5/5, Hi Load 16/60 Superdex 200 pg, PD-10, Percoll and antirabbit Ig linked to horseradish peroxidase (Amersham Pharmacia Biotech).
14. ES medium supplemented with 5% (v/v) fetal bovine serum, L-glutamate (292 mg/L), penicillin G (100 U/mL, streptomycin sulfate (100 μg/mL) and NaHCO3 (2.2 g/L).
15. 0.25 *M* sucrose.
16. 0.25 *M* sucrose containing 80, 35, or 17 %(weight/volume) metrizamide (Sigma Aldrich), or 6 % Percoll.
17. Buffer A: 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 0.25 *M* sucrose, 1 m*M* (EDTA), 0.5% TritonX-100.
18. Buffer B: 20 m*M* Tris-HCl (pH 7.5), 0.25 *M* sucrose, 1 m*M* EDTA, 1% n-octyl-β-D-glucopyranoside.

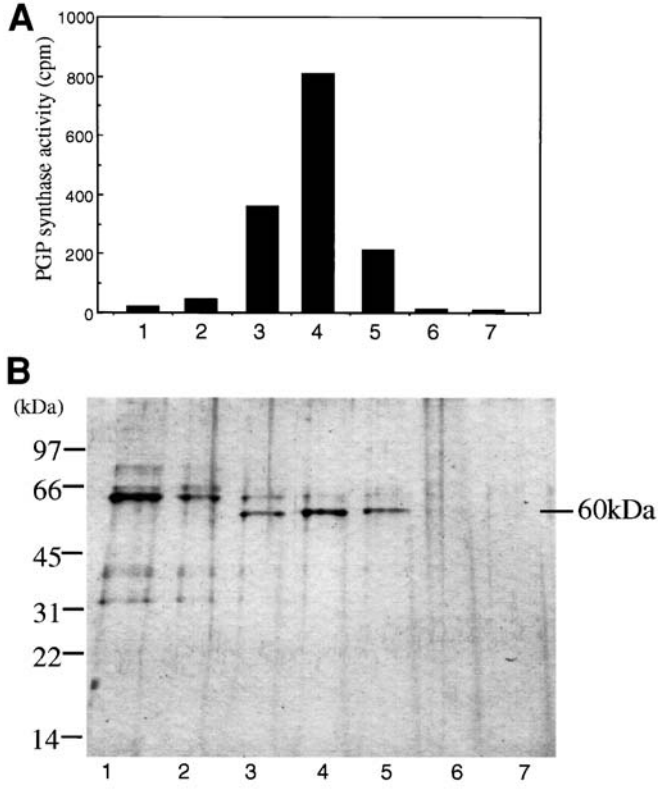

Fig. 3. Elution profiles of PGP synthase activity and 60 kDa protein on TSK-Ether chromatography. **(A)** PGP synthase activity in TSK-Ether chromatography fractions (5 μL each) was analyzed under the standard assay conditions. **(B)** Proteins in TSK-Ether chromatography fractions (100 μL each), which were used for the analysis in **(A)**, were analyzed by SDS-PAGE on a 12.5% acrylamide gel, and then visualized by silver staining. The numbers indicate the fraction numbers on TSK-Ether chromatography.

19. Buffer B containing 5 m*M* CDP and 150 m*M* NaCl.
20. Buffer B containing 5 m*M* NADH and 150 m*M* NaCl.
21. Buffer B containing 2.5 m*M* CDP-diacylglycerol.
22. Buffer B containing 20 m*M* NaCl.
23. Buffer B containing 350 m*M* NaCl.
24. Buffer C: 150 m*M* sodium phosphate (pH 6.8), 1% n-octyl-β-D-glucopyranoside (Sigma Aldrich).
25. Buffer D: 50 m*M* sodium phosphate (pH 6.8), 1% n-octyl-β-D-glucopyranoside.
26. Buffer D containing ammonium sulfate (30% saturation).
27. Buffer E: 15 m*M* sodium phosphate (pH 6.8), 1% n-octyl-β-D-glucopyranoside.

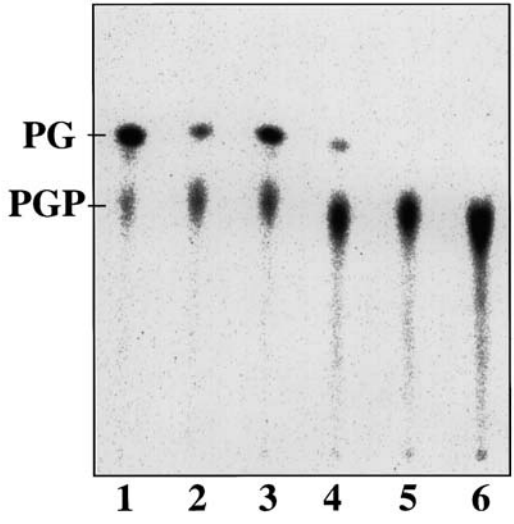

Fig. 4. Reaction products. The purified and partially purified enzyme fractions, which contained equal levels of enzyme activity (35 pmol/min), were incubated under the standard assay conditions. The radioactive lipids were separated by TLC on a 0.5 -*N* oxalic acid-impregnated Silica gel 60 plate with chloroform/methanol/10 *N* hydroxyl chloride (87:13:0.2, by volume). Lane 1, PNS; lane 2, solubilized mitochondrial proteins; lanes 3-6, pooled active fractions from the blue-Sepharose (lane 3), Mono Q HR 5/5 (lane 4), Hi Load 16/60 Superdex 200 (lane 5), and TSK-Ether (lane 6) columns.

3. Methods

3.1. Enzyme assay

PGP synthase activity was measured as aforementioned ***(2)*** with some modifications. The assay was performed at 37°C for 30 min in 50 m*M* Tris-HCl (pH 7.4), 0.25 m*M* CDP-diacylglycerol, 0.1 m*M* L-[U-^{14}C]glycerol 3-phosphate (20 mCi/mmol), 0.25% Triton X-100 and enzyme, in a total volume of 50 μL. After incubation, the lipids were extracted by the sequential addition of 600 μL of chloroform/methanol (1:2, v/v), 200 μL of chloroform, and 200 μL of phosphate-buffered saline (PBS). The lipid-containing chloroform phase was washed twice with 400 μL of chloroform/methanol/0.1 *M* KCl (3:47:48, by volume), and then the radioactivity in the chloroform phase was determined in a toluene-based scintillation mixture with a counter. For characterization of the labeled lipids, TLC on silica gel 60 plates treated with 0.5 *N* oxalic acid ***(10)*** was performed with chloroform/methanol/35% HCl (87:13:0.2, by volume), aforementioned ***(10)***.

3.2. Protein Determination

Unless otherwise indicated, protein concentrations were determined with BCA protein assay reagent using BSA as a standard.

3.3. Purification of PGP Synthase

Unless otherwise indicated, all procedures were performed at 4°C or on ice.

3.3.1. Cell Culture and Preparation of Mitochondria.

CHO-K1 cells were grown at 37°C in a glass spinner-flask containing ES medium supplemented with 5% (v/v) fetal bovine serum, L-glutamate (292 mg/L), penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL) and $NHCO_3$ (2.2 g/L), and then collected by centrifugation at 700*g* and washed twice with PBS (*see* **Note 2**). The collected 6×10^{10} cells were suspended in 60 mL of 0.25 *M* sucrose and then homogenized with a motor-driven homogenizer. The homogenate was centrifuged to remove nuclei and debris at 700*g* for 10 min, and the resultant supernatant (PNS) was centrifuged at 10,000*g* for 20 min. The resultant precipitate (crude mitochondria) was suspended in 0.25 *M* sucrose.

3.3.2. Purification and Solubilization of Mitochondria

Mitochondria were further purified by hybrid Percoll-metrizamide discontinuous density gradient centrifugation as described ***(11)***. All solutions used for this centrifugation were in 0.25 *M* sucrose and expressed as % (weight/volume). Gradients were prepared in cellulose nitrate centrifuge tubes for a Beckman SW-41 rotor as follows.

1. Overlay 2 mL of 17% metrizamide on a 1.8-mL cushion of 35% metrizamide.
2. Onto the 17% metrizamide layer, overlay 3.5 mL of 6% Percoll.
3. Gently fill the tube with 4.2 mL crude mitochondria.
4. Balance with 0.25 *M* sucrose

Then the tubes were centrifuged at 20,000 rpm (Beckman SW-41 rotor) for 20 min with the slowest acceleration and braking rates. The mitochondria formed a band at the 17/35 % metrizamide interface. A piece of Scotch Tape was attached to the outside of each centrifuge tube. An 18 gauge needle with syringe was inserted and the 17/35% metrizamide interface was recovered. Because the 6% Percoll/17% metrizamide interface also contained a rather large amount of mitochondria, the 6% Percoll/17% metrizamide interface mitochondria were recovered with a needle, and the mitochondria were further purified from the interface by second centrifugation. The second gradients were prepared and centrifuged as follows.

1. In an SW-41 tube, mix 1.125 mL of the 6% Percoll/17% metrizamide interface with 0.875 mL of 80% metrizamide. This brings the metrizamide concentration to 35%.
2. Overlay 2 ml of 17% metrizamide.
3. Overlay 2 ml of 5% metrizamide.
4. Fill the tube with 0.25 *M* sucrose.
5. Centrifuge at 20,000 rpm (Beckman SW-41 rotor) for 20 min with the slowest acceleration and braking rates.
6. Collect the 17/35% metrizamide interface as aforementioned.

The 17/35% metrizamide interface prepared from the first or second centrifugation was placed in an SW-28 tube, and then 38.5 mL of 0.25 *M* sucrose was added to the tube. To remove metrizamide from the mitochondria fraction, the diluted mitochondria were centrifuged 20,000 rpm (Beckman SW-28 rotor) for 20 min, and then 38.5 mL 0.25 *M* sucrose was added to the precipitate followed by centrifugation again. Then the precipitate (purified mitochondria) was adjusted to 4 mg protein/mL of 5 m*M* Tris-HCl (pH 7.5), 0.1 *M* sucrose, 1 m*M* EDTA, and 1% (v/v) Triton X-100, and then sonically disrupted. The disrupted mitochondria were centrifuged at 100,000*g* for 60 min, and the supernatant (solubilized mitochondrial proteins) was stored at −80°C for further purification. The solubilization efficiency for PGP synthase was approx 80% (*see* **Table 1**) (*see* **Note 3**). The PGP synthase in the solubilized mitochondrial protein was purified by successive chromatography on blue-Sepharose, Mono Q ion exchange, Superdex 200 gel filtration, and TSK-Ether hydrophobic columns (*see* later).

3.3.3. Blue-Sepharose

Half the solubilized mitochondrial protein was passed (flow rate, 4 mL/h) through a blue-Sepharose (10 mL) column equilibrated with Buffer A. Subsequently, the column was sequentially washed with 25 mL Buffer B containing 5 m*M* CDP and 150 m*M* NaCl, and 25 mL of Buffer B containing 5 m*M* NADH and 150 m*M* NaCl (*see* **Note 3**). After this washing, the column was equilibrated with Buffer B containing 2.5 m*M* CDP-diacylglycerol and incubated at 4°C for about 12 h, and then PGP synthase activity was eluted with Buffer B containing 2.5 m*M* CDP-diacylglycerol.

3.3.4. Mono Q

The PGP synthase fraction was diluted with Buffer B to reduce the NaCl concentration to less than 20 m*M*. The diluted fraction was applied (flow rate, 0.5 mL/min) to a Mono Q HR 5/5 column equilibrated with Buffer B. After washing with 25 mL Buffer B containing 20 m*M* NaCl, PGP synthase was eluted with 30 mL of a linear NaCl gradient (20–350 m*M*) in Buffer B.

3.3.5. Superdex 200

The PGP synthase fractions recovered from the Mono Q column were applied (flow rate, 0.4 mL/min) to a Hi Load 16/60 Superdex 200 column, and eluted with Buffer C.

3.3.6. TSK-Ether

Solid ammonium sulfate was added to the PGP synthase fraction recovered from the Superdex 200 column to a final saturation level of 30% at room temperature, and then the pooled active fractions were applied (flow rate, 1 mL/min) to a TSK-Ether 5PW (TOSO) column at room temperature. After washing with 8 mL buffer D (50 m*M* sodium phosphate (pH 6.8), 1% n-octyl-β-D-glucopyranoside) containing ammonium sulfate (30% saturation), PGP synthase was eluted with 30 mL of Buffer D (flow rate, 1 mL/min) containing a linear ammonium sulfate gradient (30–0% saturation) at room temperature. The fractions eluted from the TSK-Ether column were desalted with PD-10 in Buffer E (15 m*M* sodium phosphate (pH 6.8), 1% n-octyl-β-D-glucopyranoside). The desalted fractions were stored at −80°C for further analysis.

4. Notes

1. Although we have established a purification procedure for PGP synthase from CHO-K1 cells, the amount of the purified protein was too small to analyze its structure. Approx 0.32 μg of the enzyme was purified from the cells collected from more than 150 L of culture (*see* **Table 1**). We have established a CHO strain, CHO/PGS1, that overexpresses PGP synthase through the introduction of an expression construct encoding CHO PGP synthase, PGS1 ***(9)***. The PGP synthase activity in the cell extract of CHO/PGS1 was 7.5-fold higher than that in the case of wild-type CHO-K1 cells, and the expression level of the PGS1 protein in the cell extract of CHO/PGS1 was also much higher than that in the case of wild-type CHO-K1 cells (*see* **Fig. 5**). CHO/PGS1 might be a good enzyme source for PGP synthase purification.
2. It is important to choose an enzyme source in which the content of the target enzyme is high. In our case, we screened several animal tissues and mammalian cell lines for the purification of PGP synthase. Unexpectedly, the specific activity of PGP synthase in the CHO mitochondria fraction was the highest among the samples we tested (*see* **Fig. 6**). Although we do not know why the activity of PGP synthase in CHO cells is high, we believe that one of the reasons why we succeeded in purifying the enzyme is the choice of CHO cells as the enzyme source.
3. Triton X-100 was used for the solubilization of PGP synthase from mitochondrial membranes, and the existence of the detergent in the buffer was essential for retaining the activity of the solubilized enzyme. Because the purification fold did not

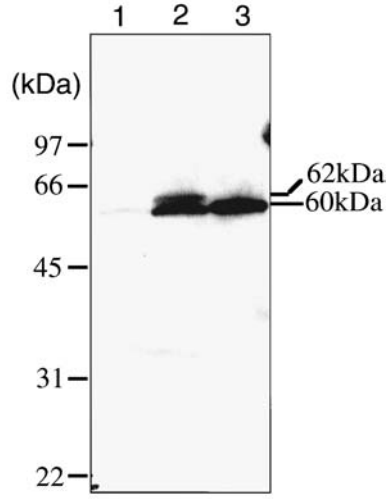

Fig. 5. Western blot analysis of a CHO/PGS1 cell lysate with anti-(PGS1 protein) antibodies. Sonically disrupted cell lysates of CHO-K1 (50 μg, lane 1) and CHO/cPGS1 (50 μg, lane 2) cells, and the purified PGP synthase (0.6 ng, lane 3) were separated by SDS-PAGE on a 10% acrylamide gel, and then analyzed by Western blotting with an anti-(PGS1 protein) antibody ***(9)*** as the first antibody and antirabbit Ig linked to horseradish peroxidase as the second antibody.

increase in the further purification steps in the presence of Triton X-100, we replaced the buffer containing 1% Triton X-100 with that containing 1% n-octyl-β-D-glucopyranoside for the step of blue-Sepharose column chromatography. Although n-octyl-β-D-glucopyranoside could not solubilize PGP synthase from mitochondrial membranes, it was as equally effective as Triton X-100 for retaining the activity of the solubilized PGP synthase.

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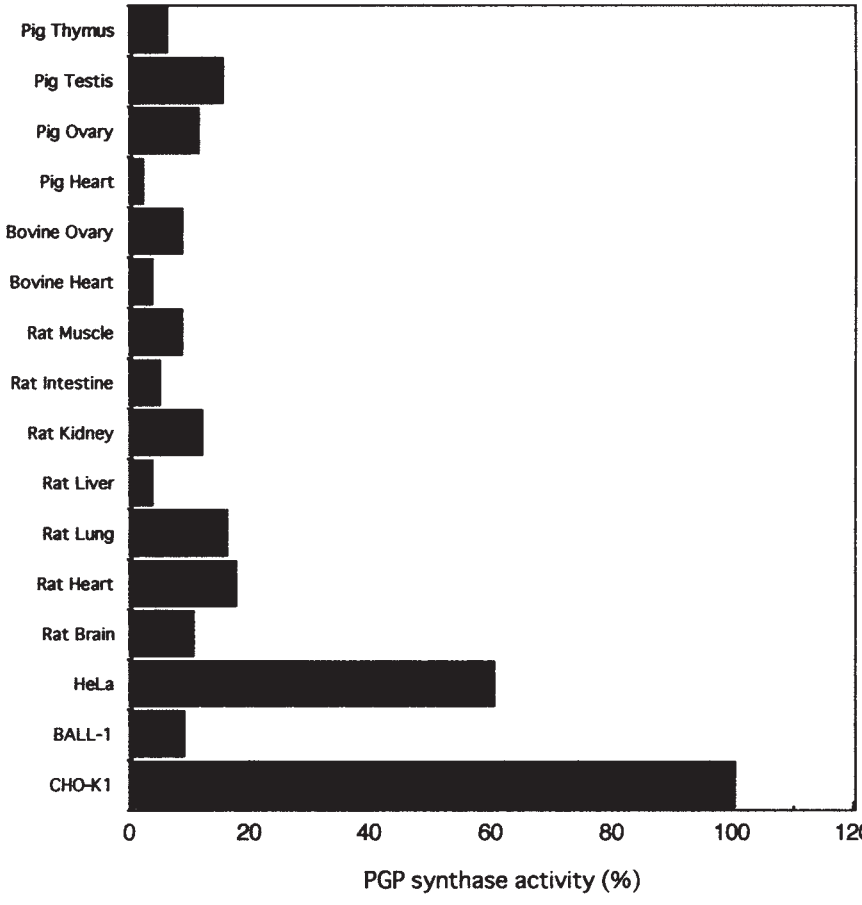

Fig. 6. Comparision of PGP synthase activity in mitochondria fraction prepared from animal tissues and cultured cell lines. Animal tissues and cultured cells were homogenized, and nuclei and debris were removed by centrifugation (700*g* for 10 min), and the resultant supernatant was centrifuged at 10,000g for 20 min. The resultant precipitate (crude mitochondria) was suspended in 3 m*M* Tris-HCl (pH 7.5) containing 0.25 *M* sucrose, and then PGP synthase activity in 25 μg of mitochondrial protein was determined. The activity is relative to that obtained in the mitochondria fraction of CHO-K1 cells (100%).

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16

Purification of Pancreastatin Receptor from Rat Liver Membranes

Víctor Sánchez-Margalet, José Santos-Alvarez, and Sandra Díaz-Troya

1. Introduction

Purification of a protein is an important step in the biochemical and structural characterization of the molecule. When the protein to be purified has not been cloned, the purification to homogeneity may be useful to find out some information about the sequence. Because G protein-coupled receptors (GPCRs) play important roles in many biological processes, the purification and characterization of a new member of GPCR is an important task to search for new pharmaceutical tools in pathophysiology ***(1–3)***. This may be the case for the receptor of the chromogranin A (CGA)-derived peptide pancreastatin (PST) ***(4–6)***.

Even though the physiological role of PST has not been unravelled yet, many different effects have been described regarding the modulation of endocrine and exocrine secretion from different glands, and the regulation of glucose, lipids and protein metabolism ***(5,6)***. Autocrine, paracrine, and endocrine actions have been observed in a variety of systems. The metabolic actions of PST have been more thoroughly studied and we already know some of the molecular mechanisms whereby PST modulate glucose, lipid, and protein metabolism, with a general counterregulatory effect of insulin action ***(7–10)***. One of the hallmarks to confirm the endocrine nature of a peptide is the presence of specific receptors in the plasma membrane. In this line, PST binding sites have been characterized in rat liver, adipose, and heart membranes ***(11–14)***. Evidence from different approaches have been provided suggesting the coupling of PST receptor with G proteins ***(11–17)***. Therefore, our working hypothesis is that PST receptor is a

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GPCR, which should be fully characterized and analyzed to finally elucidate the sequence and structure of the protein.

Binding data obtained using radioiodinated rat PST in membranes from different tissues suggested the presence of specific receptors of high affinity for PST. The PST binding fulfils all the criteria for membrane receptors: it is temperature-, time-, and pH-dependent, and it is saturable and reversible ***(11–14)***. Besides, the binding of the ligand is very sensitive to the PST sequence, further suggesting the specificity of the receptor. Thus, PST from species with low percentage of sequence homology with rat PST, such as human or porcine PST, showed a low affinity in the studies of radioligand displacement ***(18)***. Analysis of binding data under equilibrium conditions showed similar affinity values in rat liver, HTC rat hepatoma, rat adipose, and heart membranes ***(11–14, 18)***, indicating the presence of a single site with a Kd ranging 0.2-1 n*M*. This range of Kd correlates well with the ED50 obtained for PST effects in hepatocytes and adipocytes, and is in accordance with PST levels found in pig ***(19)*** and human plasma ***(20,21)***. Besides, these values are comparable to those obtained for most peptidic hormone receptors ***(22)***. Therefore, the affinity of the putative PST receptor is consistent with a possible physiological, as well as pathophysiological role of this regulatory peptide.

On the other hand, different concentrations of binding sites have been found depending on the target tissue, from 5 fmol/mg of protein in adipose tissue ***(12)*** to 34 fmol/mg protein in heart membranes ***(14)***, with an intermediate Bmax of 15 fmol/mg of protein in rat liver membranes ***(18)***. These binding data give a stimated number of binding sites of 1000–5000 per cell.

Active PST receptors have been solubilized and characterized from rat liver membranes ***(11)***. Molecular analysis of the solubilized receptor by covalent crosslinking and further identification on Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated a single band of 85 kDa. Gel filtration studies of the solubilized receptors confirmed the 80 kDa molecular mass of the PST receptor. Besides, the solubilized receptor is a glycoprotein that can be specifically bound to the wheat-germ agglutinin (WGA) lectin ***(11)***.

Taking advantage of the glycoprotein nature of the receptor, a two-step procedure has been recently employed as a purification strategy ***(23)***. Thus, WGA semipurification followed by affinity purification using a biotinylated PST analog has led to the purification of PST receptors to homogeneity. This PST analog was tested for binding to liver membranes and data showed comparable results to those of native PST binding. The PST receptor can be purified as an 80-kDa monomeric glycoprotein physically associated with a $G\alpha_{q/11}$ protein. The scale-up of the purification process may yield sufficient amount of receptor proteins to undertake microsequencing in the near future.

In the present chapter, we present a description of the PST purification from rat liver. We are providing, in more detail, the practical procedures of the purifi-

cation strategy of PST receptor from solubilized rat liver membranes, that we have followed, as well as the problems that we have encountered and how we have solved them.

2. Materials

2.1. Membrane Preparation

1. Rats (albino Wistar, 170–220 g) were from the animal facility (Medical School of Seville, Spain).
2. Phosphate-buffered Saline (PBS) containing 1 m*M* ethylenediamine tetraacetic acid (EDTA), kept at 4°C. HEPES, EDTA, NaCl, MgCl, KCl, Triton X-100, glycerol and the protease inhibitors (calpain inhibitor-II, bestatin and trypsin inhibitor [from soybean]) were from Roche Molecular Biochemicals (Barcelona, Spain).
3. Homogenization buffer: 10 m*M* HEPES (pH 7.4), 0.1 m*M* EDTA, and protease inhibitors (bacitracin, benzamidine, leupeptin, TLCK, phenyl methyl sulfonyl fluoride [PMSF], pepstatin, aprotinin were from Sigma (Madrid, Spain) and were used at the final concentration recommended by the manufacturer. Protease inhibitors should be added immediately before use.
4. Washing buffer: 20 m*M* HEPES (pH 7.4), and protease inhibitors (*see* **step 3**).

2.2. Solubilization of Rat Liver Membranes

1. Solubilization buffer: 25 m*M* HEPES (pH 7.4), 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* KCl, 1% Triton X-100 (v/v) and protease inhibitors at the recommended final concentration. Protease inhibitors should be added immediately before use.

2.3. Affinity Chromatography

1. Wheat germ agglutinin (WGA) coupled to agarose and streptavidin immobilized in Sepharose was from Amersham Pharmacia (Barcelona, Spain).
2. Washing buffer for both columns was the same as solubilization buffer but with a lower Triton X-100 concentration (0.1%).
3. Column buffer: 25 m*M* HEPES (pH 7.4), 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* KCl, 0.3 *M* N-acetylglucosamine (Sigma, Madrid), 1% Triton X-100 (v/v) and protease inhibitors at the recommended final concentration.
4. Biotin-PST was from Peninsula Laboratories (Merseyside, U.K.).

2.4. Ligand Affinity Purification

1. Column buffer: *see* **Subheading 2.3.**
2. Elution buffer: column buffer, adjusted to pH 5.5 with HCl.

3. Methods

3.1. Membrane Preparation (see Note 1)

1. Rats were sacrificed with a guillotine.
2. Livers ***(4–5)*** were then washed with cold PBS containing 1 m*M* EDTA via portal and cava vein.

3. Livers were cut into pieces with a scissors and homogenized with cold and freshly made homogenization buffer with the cocktail of protease inhibitors in a blender.
4. The homogenate was filtered through a gauze mesh and further homogenated by a manual glass potter (three strokes).
5. The liver homogenate was then centrifuged at 50,000*g* for 30 min to pellet crude membranes.
6. The membranes were then washed with washing buffer, pelleted by centrifugation (50,000*g*, 20 min) and stored at −80°C until use. The average yield of membrane protein was 100–120 mg per rat. Membranes can be stored at −80°C for up to 6 mo.

3.2. Membrane Solubilization (see Fig. 1)

1. Rat liver membranes (400 mg of protein) were solubilized with 1% Triton X-100. Solubilization was carried out by incubating membranes (14 mg/mL) in freshly made solubilization buffer with the protease inhibitors cocktail for 1 h at 4°C rotating end over end.
2. The membrane suspension was then centrifuged at 100,000*g* at 4°C for 1 h, and the supernatant used immediately. The yield of membrane protein solubilization is about 50%, yielding a final concentration of 7 mg/mL.

3.3. Wheat Germ Affinity Chromatography (see Fig. 1)

We have previously demonstrated that PST receptors can be semipurified by lectin chromatography *(11–15)*.

1. Solubilized membranes (200 mg of protein at 5 mg/ml) were incubated with agarose-WGA (4 mL) for 3 h at 4°C, with rotation.
2. The mixture was then packed into a 20 × 3-cm-chromatographic column, and the column was washed 2 × with 20 vol of solubilization buffer with 0.1% Triton X-100.
3. The glycoproteins were eluted at 0.3 mL/min with the same buffer containing 0.3 *M* N-acetyl-D-glucosamine. Fractions (1 mL) were collected, and protein concentration was determined in each fraction to localize the fractions with the eluted glycoprotein. The selected fractions were then pooled for further purification.

3.4. Ligand Affinity Purification (see Fig. 1)

Biotinylated analogs have been previously used for ligand affinity purification of different receptors *(24,25)*.

1. The ligand affinity matrix was prepared by incubating 100 nmol bio-PST with 1 mL streptavidin-Sepharose, in column buffer for 1 h at 4°C.
2. The matrix was then packed into a 15 × 1-cm column.
3. The column was then washed 3× with 20 vol of column buffer. WGA semipurified extracts (2.5 mg of protein), corresponding to the pooled fractions of the WGA elu-

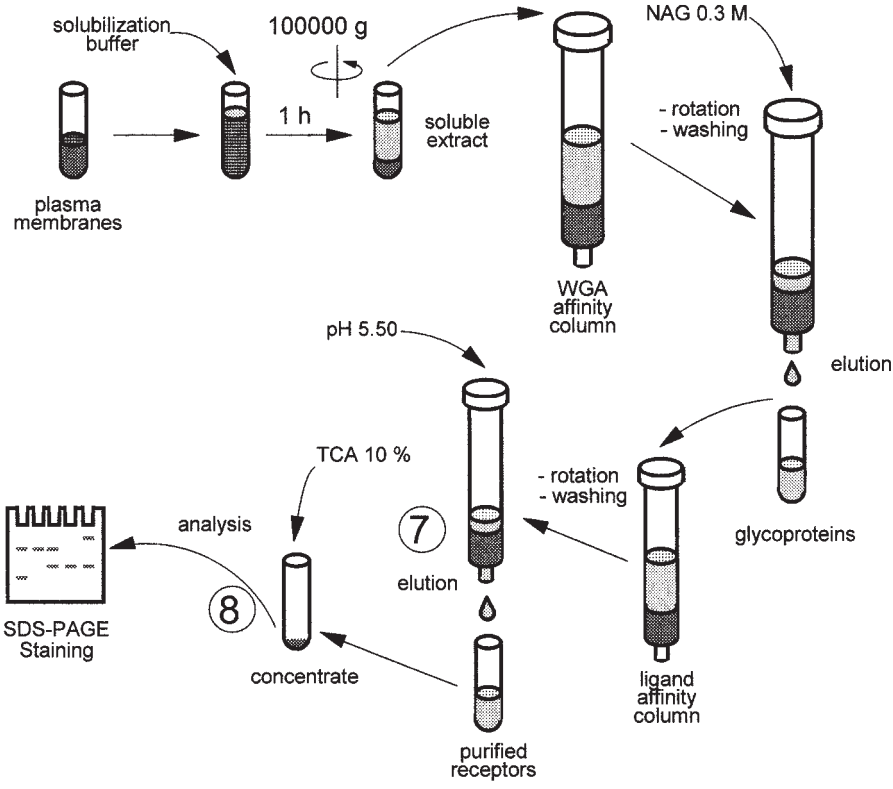

Fig. 1. Scheme of the sequential steps of PST purification from rat liver membranes.

tion, (about 10 mL) were incubated for 18 h with Bio-PST, which had been previously bound to streptavidin on Sepharose beads (*see* **Note 2**).

4. The mixture was then packed into a 25 × 1-cm-chromatographic column.
5. The column was washed 3 × with 20 vol of the same buffer used in WGA chromatography, and eluted with the same buffer at pH 5.5.
6. Fractions (2 mL) were then collected and concentrated by TCA (10%) precipitation (*see* **Note 3**), followed by SDS-PAGE (8–16%). The gel was then fixed and silver stained (*see* **Fig. 2**). A major band at 80 kDa was eluted at pH 5.5 (2 column) volumes. A higher molecular weight of about 150–160 kDa is also observed, as well as a 40–43-kDa component. Further washing of the column did not show any protein content, demonstrating that all the material bound to the column was eluted.

4. Notes

1. The major technical problem of the receptor purification is the protein degradation, which should be kept to the minimum. The following tips should be kept into mind. Keep the buffers and materials always at 4°C. For membrane preparation, the con-

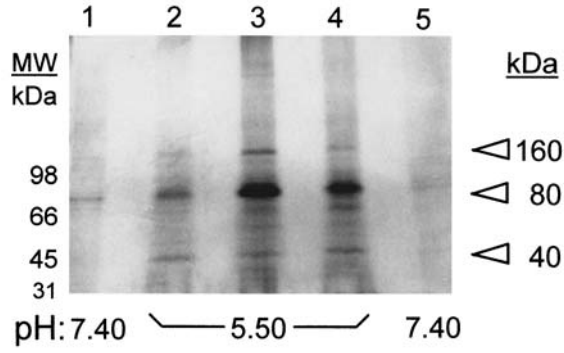

Fig. 2. Silver stained gel (SDS-PAGE) with the analyzed samples corresponding to the elution fractions of the ligand affinity column. The first lane corresponds to concentrated 2 mL washing buffer passed through the column. Lanes 2–4 are concentrated elution fractions (2 mL) using low pH (5.5). Lane 5 is concentrated washing buffer (pH 7.4) passed through the column after the elution.

tainer of the blender may be previously frozen, to avoid overheating during the homogenization. Add the protease inhibitors at the last moment.

2. When there is a long-time incubation period (e.g., the ligand affinity column), further protease inhibitors should be added, at intermediate times. The stored liver membranes should be used within few weeks. Once the solubilization has finished, immediately continue with the WGA chromatography, followed by the ligand affinity chromatography. One can even avoid overnight incubation with the ligand affinity matrix by cutting the incubation time to 3 h, but this means having a very long work day.
3. As the trichlorocetic acid (TCA) precipitated fractions are very acid, the pH should be increased by adding 3–5 μL of 100 m*M* Tris-HCl (pH 9.0). Otherwise, samples do not run properly in the SDS-PAGE.

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Purification, Reconstitution, and Functional Characterization of Zinc Transporter from Rat Renal Brush Border Membranes

Rajendra Prasad

1. Introduction

Zinc is an integral component of a wide variety of functional proteins, enzymes, and transcription factors where it exerts specific actions over a wide range of physiological processes such as growth, development, and functioning of the endocrine, immune, and nervous systems ***(1–4)***. Zinc is also involved in stabilizing membrane structure, and in protection at the cellular level by preventing lipid peroxidation and reducing free radical formation ***(5)***. The primary site of zinc regulation in mammals is intestinal absorption. After being absorbed, zinc is bound to albumin in the circulation, where it is maintained within a narrow range (1 μg/mL) in mammals. Most of the zinc is taken up by the liver before being redistributed to other organs. The primary routes of zinc excretion are via pancreatic, biliary, and intestinal secretions. A very small amount of zinc is excreted by the kidney, as most of the zinc from the glomerular filtrate gets reabsorbed in the renal tubular system.

At the cellular level, specific transporters are presumably responsible for uptake and maintenance of intracellular concentration of zinc. Regulation of the number, activity, and cellular location of these transporters is likely to play an important role in zinc homeostasis. Patients with severe renal failure and nephrotic syndrome have been found to be associated with hypozincemia ***(6,7)***. In addition, patients with stress, trauma, chronic renal failure, and a variety of malignancies have low serum zinc levels and high urinary zinc excretion ***(8)***. All of these zinc deficiencies are currently thought to be associated with a defect in renal tubular zinc reabsorption. Therefore, reabsorption of zinc across the brush

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border membrane in renal tubular cells is equally important to the adequate intake and absorption of zinc in the intestine. Genetic defects of zinc deficiency include *Acrodermatitis enteropathica* in humans ***(8)*** and Adema (Trait A-46) disease in cattle, which is inherited as an autosomal recessive trait ***(9)***.

The intestinal and renal absorptive cells are polar in nature. The membrane exposed to the renal lumen (BBM) is functionally and structurally distinct from the basolateral membrane, which is in contact with extracellular fluid (*see* **Fig. 1**). The BBM is the first barrier encountered by various solutes during absorption and reabsorption in intestine and kidney, respectively. Renal brush border membranes continue to be the object of numerous investigations. The reasons for their wide experimental use stem from their varied enzymatic and transport activities particularly regarding absorption and secretion. Also, they can easily be isolated in relatively pure form because a sufficiently high proportion of the membranes are isolated as sealed vesicles that have been extensively used as models for transport measurements.

The aim of this chapter is to provide an overview of the problems and the procedures involved in the isolation, purification, and functional reconstitution of zinc transporter from renal tubule brush border membrane of the rat.

1.1. Isolation of Renal Brush Border Membrane

The epithelium of the renal proximal tubules that mediates vectorial net transport of solutes and fluid is characterized by cells with determined polarity. This asymmetry is evident ultrastructurally by the differentiation of the plasma membrane into two distinct components, the apical brush border and antiluminal basal lateral membranes, and functionally by differences in the enzyme composition of the two membranes, as well as in the mechanisms by which substances enter and exit the cell.

Studies on the structure and function of the brush border membranes have been greatly aided by the development of rapid methods for their isolation ***(10,11)***. These membranes are routinely prepared by homogenizing and precipitating most of the undesired organelles and basolateral membranes with divalent cations, i.e., Ca^{2+}, Mg^{2+}, and Mn^{2+} ***(12,13)***. These membrane vesicles display a right-side orientation as evidenced by electron microscopy.

1.2. Solubilization of Brush Border Membrane Proteins

Kinetic characterization of the transport system in intact renal brush border membrane vesicles (BBMV) is the most important step before proceeding to the purification of its transmembrane transporter. Measurements of kinetic constants and other physicochemical properties of transport assist further in the purification by serving as reference values. The kinetic characterization of solute binding to BBM, and its isolation and functional reconstitution into

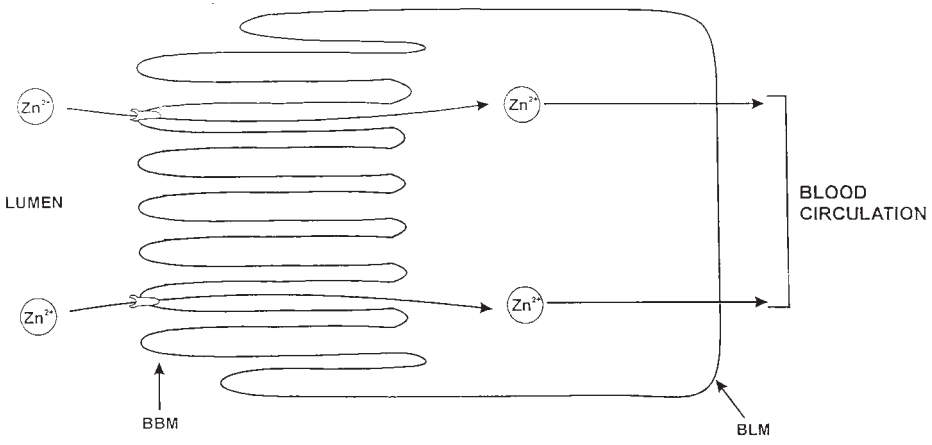

Fig. 1. Renal epithelial cell. The first barrier in the transport of solutes is the brush border membrane (BBM) BLM; basolateral membrane.

liposomes are intrinsic in better understanding the molecular basis of the transporter.

The success of solubilization, purification, and reconstitution is often determined by the choice of a suitable detergent. In general, no single detergent has emerged as the best choice in all cases. The reasons for these are as follows:

1. Genuine differences in the effects on specific proteins.
2. Lack of systematic solubilization and reconstitution strategies.
3. Complexity of the interaction between molecules of such distinct chemical structure as proteins, lipids, and detergents.
4. Consequently, the selection of a particular detergent for solubilization and reconstitution is based on precedent and empirical factors rather than on scientific principles.

The purification and characterization of membrane proteins presents several problems that are not encountered with soluble proteins. The membrane proteins are classified as extrinsic or intrinsic proteins according to the method used to solubilize them ***(14)***. Characterization and purification of intrinsic proteins requires that they are extracted from the membrane lipid bilayer and inserted into detergent micelles.

The critical micelle concentrations for various detergents are mentioned in **Table 1**. Nonionic detergents with high CMC have been used for the solubilization and purification of a majority of carrier proteins. A method was employed for the reconstitution of zinc transporter solubilized by nonionic detergent with high critical micellar concentration because of the following properties.

Table 1
Chemical and Physical Properties of Commonly Used Detergents

Detergent	Mol. wt. monomer	Mol. wt. micelle	CMC[a]		Refs
			% (w/v)	mM	
Sodium dodecyl sulfate (SDS)	288	18,000	0.23	8.0	***14,36***
Deoxy Cholate	432	4200	0.21	5.0	***37***
Lyso PC (C_{16})	495	92,000	0.0004	0.007	***14,36***
CHAPS	615	6150	0.49	1.4	***37***
Zwittergent 3–14	364	30,000	0.0011	0.30	***37***
Octyl glucoside	292	8000	0.73	23	***37***
$C_{12}E_8$	542	65,000	0.005	0.087	***37***
Lubrol PX	582	64,000	0.006	0.1	***37***
Triton X-100	650	90,000	0.021	0.3	***14,36***
Tween-80	1310	76,000	0.002	0.012	***38***

[a]Determined at 20–25°C.

1. It is nonionic.
2. It has a low molecular weight.
3. It has a high CMC.
4. It is easily dialyzable ***(15)***.

In the present studies, proteins are solubilized by n-octylglucoside, and functional reconstitution is achieved using the same detergent.

It is very difficult to directly relate the CMC of a detergent to its potential for interacting with the solubilizing lipid-protein bilayer. The level of solubilization depends on both the lipid concentration and the concentration of detergent micelles. A measure of the effective concentration of detergent would be useful, and attempts have been made to derive such a parameter. The most useful of these is a the Q (rho) parameter, introduced by Rinvay and Metzger ***(15)***, where

$$Q = \frac{[Detergent] - CMC_{\text{eff}}}{[Phopholipid]}$$

The CMC_{eff} describes the CMC determined under specific experimental conditions. The CMC_{eff} is preferred to the CMC, as the latter is often lowered in the presence of lipids and proteins ***(14)***, but in practice, the literature values of CMCs are used (*see* **Table 1**). The Q-factor attempts to describe the molar ratio of micellar detergent to phospholipid employed. An increase in solubilization is predicted to occur as the Q-parameter is raised.

1.3. Purification of Solubilized Proteins

All of the major types of chromatography are routinely carried out using the column mode. The apparatus and general techniques used for various column chromatography (i.e., ion exchange, hydrophobic interaction affinity, covalent, metal chetale, chromatofocusing, and exclusion) have much in common. The characteristics of the various forms of chromatography used in protein purification are summarized in **Table 2**. There is no universal prescription for the order in which the various techniques should be applied for the purification of a particular protein. Ion exchange and hydrophobic interaction chromatography have been developed to facilitate protein purification, and are commonly used after the early crude fractionation stages. The development of a successful protocol involves a considerable amount of trial and error and the use of a number of small-scale pilot experiments to assess the potential of each proposed stage. This, in turn, relies upon the availability of a sensitive and specific functional reconstitution of the transporter system. In general, it is advantageous to design a protocol that progressively exploits different properties of the test protein.

Table 2
Summary of Chromatographic Techniques Commonly Used in Protein Purification

Technique	Property exploited	Resolution	Practical points
*Hydrophobic interaction	Hydrophobicity	Medium	Can cope with high ionic strength. Sample fractions are of varying pH and/or ionic strength. Commonly used in early stages of purification protocol.
*Ion exchange	Charge	Medium	Sample ionic strength must be low. Fractions are of varying pH and/or ionic strength. Commonly used in early stages of purification.
*Affinity	Biological function	High	Limited by availability of immubilized ligand. Elution may denature protein. Commonly used towards end of purification protocol.
*Chromatofocussing	Charge and pI	High-medium	Sample ionic strength must be low. Fractions contaminated with ampholytes.
*Covalent	Thiol group	High	Specific for thiol containing proteins.
*Exclusion	Molecular size	Low	Provide information about molecular weight. Used for desalting protein samples. Commonly used as a final stage of purification.

1.4. Functional Reconstitution of Purified Proteins

Reconstitution of a membrane protein typically begins with a mixed micellar solution of purified protein, detergent, and lipid. Proteoliposome formation requires removal of the detergent from the mixed micelles so that a phospholipid bilayer can form spontaneously. The most common method of detergent removal is dialysis. The micellar solution is enclosed within a dialysis bag and dialyzed against a large volume of detergent-free buffer, usually in the cold conditions. A complete study of any reconstitution protocol requires some knowledge of the morphology of the products. Ideally, the reconstituted vesicles should be homogeneous in shape, size, and protein distribution. It is especially important to assay bilayer permeabilities when protein orientation is to be characterized or when vectorial translocation is to be measured. Monoclonal antibodies specified for external or internal domains of the membrane protein can be used to measure sidedness.

2. Materials

2.1. Animals

1. Male adult albino rats of Wistar strain.
2. Five- to six-month-old healthy rabbits (New Zealand white strain).

2.2. Preparation of Brush Border Membrane Vesicles

1. Homogenizing buffer: 50 m*M* mannitol, 15 m*M* HEPES–KOH (pH 7.0)
2. 0.4 *M* $MnCl_2$.
3. Reconstitution buffer: 300 m*M* mannitol, 15 m*M* HEPES-KOH (pH 6.9). Store the solutions at 4°C.
4. Potter-Elvehjem homogenizer.
5. Polytron sonifier.

2.2.1. Purity of Brush Border Membrane

2.2.1.1. Alkaline Phosphatase (EC 3.1.3.1) Assay

1. Buffered Substrate: 0.5 *M* glycine buffer, 5.5 m*M* p-nitrophenyl phosphate, (pH 10.5). Dissolve 375 mg glycine, 10 mg $MgCl_2$, and 165 mg p-nitrophenyl phosphate (sodium salt) in 42.0 mL of 0.1 *N* NaOH, dilute to 100 mL with ddH_2O, and adjust the pH to 10.5.
2. 5.5 m*M* p-nitrophenol (standard solution): Dissolve 13.9 mg p-nitrophenol in 100 mL distilled water (1 μmol/mL). Prepare the solution fresh daily.
3. 0.1 *M* NaOH.

2.2.1.2. Maltase Assay (EC 3.2.1.20)

1. 50 m*M* phosphate buffer (pH 6.0).
2. Glucose oxidase-peroxidase reagent kit: Glucose oxidase-peroxidase reagent (Sigma) is dissolved in 1 L of 0.5 *M* Tris-HCl, pH 7.0, containing 2.0 mL of Triton X–100.
3. Substrate: 0.1 *M* maltose in 50 m*M* phosphate buffer (pH 6.0)

2.2.1.3. Na^+/K^+ ATPase (EC 3.6.1.3)

1. ATPase reaction buffer: 5 m*M* ATP in 30 m*M* Tris-HCl buffer, pH 7.1, containing 20 m*M* KCl and 7.5 m*M* $MgCl_2$.
2. 40 m*M* Ouabain (stock), store at – 20°C.
3. 2.5 *M* perchloric acid.

2.2.1.4. Inorganic Phosphorus Assay Reagent

1. Ammonium molybdate. Dissolve 25 g of ammonium molybdate in 200 mL of ddH_2O, add 300 mL of 10 *N* H_2SO_4 and make the final volume 1 L with ddH_2O.
2. 1-Amino-2-naphthol-4-sulphonic acid (ANSA): Dissolve 0.5 gm of ANSA in 195 mL of 15% sodium bisulfite and add 5.0 mL of 20% sodium sulfite.
3. Phosphate standards (5–40 μg).

2.2.1.5. Protein Assay Reagent

1. Bradford reagent stock: 100 mg of Coomasie brilliant blue.
2. 95% ethanol.
3. 85% orthophosphoric acid.
4. Bradford working reagent (BWR). Stock solution (3.0 mL) is diluted to 10.0 mL with ddH_2O and filtered through Whatman No. 10 filter paper before use.
5. Standard stock (BSA—1 mg/mL) working standard (10 μg to 200 μg).
6. Microplate reader.

2.2.1.6. Electron Microscopy Reagents

1. 3% glutaraldehyde in 0.1 *M* cacodylate (pH 7.2).
2. 1% Agar.
3. 1% Osmium tetroxide.
4. Millonig's buffer: 0.02 sodium dihydrogen orthophosphate and 0.02 sodium hydroxide (adjust pH upto 7.4 with NaOH solution).
5. Propylene oxide.
6. Epon 812.
7. 0.5% Toluidine blue.
8. 5% alcoholic uranyl acetate (5% uranyl acetate in 70% methanol).
9. 0.31% lead citrate.
10. Sorensen phosphate buffer pH 7.4: 0.1 *M* Na_2HPO_4, and 0.1 *M* Na_2HPO_4 containing 7.5% sucrose.

2.3. Kinetic Characterization of Zinc Uptake into Renal BBMV

2.3.1. Time-Course of Zn^{2+} Uptake

1. Uptake buffer: 300 m*M* mannitol, 25 m*M* HEPES–KOH (pH 6.9) containing 1 m*M* $ZnCl_2$ and 1 μCi of radioisotope $^{65}Zn^{2+}$.
2. Membrane filters: nitrocellulose membrane (0.45 μm mesh).
3. Stop buffer: 150 m*M* KCl, 10 m*M* HEPES-KOH, (pH 6.9) containing 5 m*M* EGTA. Keep at 4°C.
4. Scintillation counter.

2.3.2. Substrate Dependence of Zinc Uptake into Renal BBMV

All the solutions used as aforementioned in **Subheading 2.3.1**. except uptake buffer with different concentrations of Zn^2 (0.1 m*M* to 2.0 m*M)*.

2.3.3. Effect of pH on Zinc Uptake

All the solutions are used as aforementioned in **Subheading 2.3.1**. except uptake buffer of various pH (2.9–6.9) as well as intravesicular pH (2.9–6.9).

1. Uptake buffer <pH 6.0 (150 m*M* KCl, 15 m*M* 2 (N-morpholino ethane sulphonic acid (MES)-KOH. Adjust pH with KOH solution). Uptake buffer >pH 6.0 (150 m*M* KCl, 15 m*M* HEPES KOH. Adjust pH with KOH solution).
2. Intravascular buffer <pH 6.0 (300 m*M* mannitol, 15 m*M* MES-KOH. pH buffer >pH 7.0 (300 m*M* mannitol, 15 m*M* HEPES-KOH).

Note: Adjust desirable pH with KOH solution.

2.3.4. Effect of Temperature on Zinc Uptake into BBMV

All the solutions are used as aforementioned in **Subheading 2.3.1**.

2.3.5. Competition and Inhibition Studies

1. All the solutions are used as aforementioned in **Subheading 2.3.2**.
2. 2 m*M* Cd^{2+} in uptake buffer.
3. Sulfhydryl blocking agent in uptake buffer (Iodoacetate [5 m*M*]).
4. Carboxyl group reacting compound 5 m*M* NN–dicyclohexyl carbodiimide (DCCD).

2.4. Purification of Major Zinc Binding Protein from Brush Border Membrane

2.4.1. Solubilization of BBM Proteins

1. Solubilization buffer: 300 m*M* mannitol, 25 m*M* HEPES—KOH (pH 7.0).
2. Radioisotope $^{65}Zn^{2+}$ (10 μCi).
3. *n*-Octylglucoside.
4. Dialysis bag (10 kDa cutoff).

2.4.2. Ion Exchange Chromatography

1. Amicon ultrafiltration assembly with 10-kD cutoff filter.
2. DEAE-Sephadex A-25 column (12 cm × 1.2 cm), preequilibrated with buffer.
2. Preequilibration buffer: 20 m*M* Tris-HCl buffer (pH 7.0) containing 1% lubrol PX.
3. Elution buffer with Cl^- gradient.
   a. 20 m*M* Tris-HCl buffer, pH 7.0, containing 1.0% lubrol PX.
   b. 20 m*M* Tris-HCl buffer, pH 7.0, containing 1.0% lubrol PX and 1.0 *M* NaCl.

2.4.3. Molecular Exclusion Chromatography

1. Sephadex G-100 column (85 cm × 1.4 cm).
2. Preequilibration buffer: 25 m*M* Tris-HCl, pH 7.0, containing 1% lubrol PX.
3. Elution buffer: same as preequilibration buffer.
4. Biobeads SM-2.

2.4.4. Hydrophobic Interaction Chromatography

1. Phenyl-sepharose column (8 cm × 0.8 cm).
2. Preequilibration buffer: 25 m*M* Tris-HCl (pH 7.0).
3. Elution buffer with *n*-octylglucoside gradient:
   a. 25 m*M* Tris-HCl (pH 7.0).
   b. 25 m*M* Tris-HCl (pH 7.0) containing 2% *n*-octylglucoside.
4. Biobeads SM-2.

2.4.5. Fast Protein Liquid Chromatography (FPLC)

1. Mono Q HR 5/5 (Pharmacia) column (50 × 5 mm).
2. Preequilibration buffer: 10 m*M* Tris-HCl (pH 8.0).
3. Elution buffer with Cl^- gradient:
   a. 10 m*M* Tris-HCl (pH 8.0).
   b. 10 m*M* Tris-HCl (pH 8.0) with 500 m*M* NaCl.

2.5. Physicochemical Properties of Purified Major Zinc-Binding Protein (MZnBP)

1. SDS-PAGE is performed with a mini-PROTEAN II electrophoresis apparatus (Bio-Rad, Mississauga, Ont. Canada) using Laemmli buffer system ***(27)***.
2. Discontinuous polyacrylamide gels (12% separating gel/ 4% stacking gel), prepared by standard procedures.
3. Sample buffer: 62.5 m*M* Tris-HCl (pH 6.8) containing 26% glycerol, 2% SDS, and 5% ß-mercaptoethanol.
4. Standard molecular weight markers. Broad range protein standard markers.

2.5.1. Silver Staining Reagents

1. Former's solution: Dissolve 0.3 g $Na_2S_2O_3$, 5 H_2O, 0.1 g $K_3Fe(CN)_6$, and 0.05 g Na_2CO_3 in 100 mL of ddH_2O. Make fresh daily.
2. $AgNO_3$ solution: Dissolve 0.405 g $AgNO_3$ in 100 mL of ddH_2O in dark bottle. Make fresh daily.

3. Developer solution: Dissolve 2.96 g Na_2CO_3 and 0.025 mL formaldehyde in 100 mL of ddH_2O. Make fresh daily.
4. Fixing solution: Mix 15 mL GAA, 40 mL methanol, and 45 mL ddH_2O.

2.5.2. Metal Displacement Assay

1. Radioisotope $^{65}Zn^{2+}$.
2. 10% slurry of chelax-100.

2.5.3. UV Absorption Study

1. Purified protein.

2.5.4. Carbohydrate Content Estimation

1. Reagent A: 5% w/v distilled phenol.
2. Reagent B: concentrated H_2SO_4.

2.6. Functional Reconstitution Study

2.6.1. Reconstitution of Purified ZnBP into Proteoliposomes

1. KHT buffer: 150 m*M* KCl, 20 mM HEPES, 15 m*M* Tris-HCl and 0.2 m*M* EGTA, (pH 6.9).
2. Purified egg yolk phosphatidylcholine.
3. Cholesterol.
4. Chloroform / methanol solution (2:1; v:v).
5. Reconstitution buffer; 300 m*M* mannitol, 25 m*M* HEPES KOH (pH 6.9).

2.6.2 Phase Contrast Microscopy for Assessment of Integrity of Proteoliposomes

1. Proteoliposomes.

2.6.3. Kinetic Characterization of Zinc Transport into the Proteoliposomes

All the solutions are used as aforementioned in **Subheading 2.3.**

2.7. Immunohistochemical Localization of Zinc Transporter

2.7.1. Raising Antisera

1. Purified protein.
2. Fruends complete adjuvant (FCA).

2.7.2. Enzyme-Linked Immunosorbent Assay (ELISA)

1. 3% BSA.
2. Polyclonal antisera.
3. Antirabbit conjugated horseraddish peroxidase.

2.7.3. Immunofluorescence Staining

1. FITC labeled antirabbit goat IgG.

3. Methods

3.1. Kidney Isolation

Sacrifice the rats under light ether anesthesia, remove both kidneys immediately, and rinse with the cold normal saline (154 m*M* NaCl) and dissect to remove medulla. Carry out all subsequent procedures at 4°C.

3.2. Preparation of Brush Border Membrane Vesicles

Rat renal cortical brush border membrane vesicles are prepared by the differential centrifugation method of Prasad et al. ***(17–19)***.

1. Dissect the cortices of kidneys, record the weight, and mince with prep blade.
2. Suspend the mince in homogenizing buffer in a ratio of 1 g of tissue to 10 mL of buffer and homogenize with three complete strokes of a Potter-Elvehjem Teflon pestle at 1000 rpm.
3. Dilute the homogenate with additional buffer to give a final ratio of 1 g of tissue to 20 mL of homogenizing buffer.
4. Transfer the suspension to a 125-mL conical flask and rehomogenize using a polytron at a power setting of 7 by pulsing three times for 30 by 30 interval.
5. Add 0.4 *M* $MnCl_2$ solution to a final concentration of 4 m*M* and stir the mixture in an ice bath for 20 min exactly.
6. Centrifuge the suspension at 4500*g* for 20 min.
7. Decant the supernatant and centrifuge at 43,000*g* for 20 min.
8. Resuspend the pelleted material representing brush border membranes in reconstitution buffer with a glass homogenizer.
9. Centrifuge the suspension at 43,000*g* for 20 min.
10. Resuspend the resulting pellet in reconstitution buffer and pass through a 26-gage needle with syringe.

3.2.1. Assessment of Purity of Isolated Brush Border Membrane (see ***Note 1***)

Check the purity of BBM by assaying marker enzymes of BBM viz alkaline phosphatase and maltase.

3.2.1.1. Alkaline Phosphatase (EC 3.1.3.1.) Assay

Alkaline phosphatase activity is determined by the method of Bergmeyer ***(20)***, using p-nitro phenyl phosphate as a substrate.

1. Incubate 1 mL of 5.5 mM p-nitrophenyl phosphate in 0.5 *M* glycine buffer (pH 10.5) for 5 min to attain a temperature of 37°C.

2. Add 100 μL of suitably diluted BBM and incubate for 15 min at 37°C.
3. Terminate the above enzyme reaction by adding 5 mL of 0.1 NaOH to each tube and mix thoroughly.
4. Measure the optical density of p-nitrophenol liberated at 420 nm.
5. Run blank and standard (p-nitrophenol concentration between 0.1–0.5 μmol) simultaneously.

3.2.1.2. Maltase (EC 3.2.1.2.) Assay

Activity of maltase, a BBM marker enzyme, is determined by measuring D-glucose liberated from maltose using glucose oxidase-peroxidase system by the method of Dahlquist ***(21)***.

1. Add 100 μL of buffered substrate to 400 μL of 50 m*M* phosphate buffer and mix thoroughly. Incubate this reaction mixture at 37°C for 5 min.
2. Add 100 μL of suitably diluted BBM and incubate at 37°C for 10 min.
3. Add 4.0 mL of glucose oxidase-peroxidase reagent.
4. Read absorbance at 560 nm after incubation at room temperature for 30 min.

3.2.1.3. Na^+/K^+ ATPase (EC 3.6.1.3.) Assay

The contamination of basolateral membrane in BBM preparations are checked by assaying Na^+/K^+ ATPase by the method of Qugley and Gotterer ***(22)***.

1. Add 100 μL of sample (BBM) to 1 mL of ATPase reaction buffer and incubate at 37°C for 30 min.
2. To measure the ouabain sensitive enzyme, add 50 μL of 40 m*M* ouabain to the assay mixture.
3. Terminate the reaction by the addition of 0.25 mL of 2.5 *M* perchloric acid and incubate on ice for 15 min. The proteins and perchlorate will be precipitated.
4. Centrifuge the tubes at 5000*g* for 10 min.
5. Estimate liberated inorganic phosphate (Pi) according to the method as described by Fiske and Subbarow ***(23)***.

3.2.1.4. Inorganic Phosphate Assay

1. Take 1 mL of clear supernatant.
2. Add 1 mL of the ammonium molybdate and 0.4 mL of ANSA reagent and mix well.
3. After 5 min, dilute content of each tube to 10 mL with ddH_2O and read absorbance at 720 nm.
4. Run blank and standard (Pi conc 5–40 mg) simultaneously.

3.2.1.5. Protein Estimation

Estimate the protein by the dye binding micro assay of Bradford ***(24)***.

1. Make the suitable dilution of the protein sample (BBM) up to 60 μL with ddH_2O.
2. Run standards (10–200 μg) as well as reagent blank simultaneously.

3. Add 140 μL of BWR solution and incubate at room temperature for 15 min.
4. Read the absorbance in an ELISA plate reader at 600 nm with differential wavelength at 450 nm.

3.2.1.6. Electron Microscopy of Renal BBMV

1. Fix final pellet of BBMV in 3% glutaraldehyde in 0.1 *M* cacodylate (pH 7.2) for 24 h at 2–8°C.
2. Add 1–2 mL of 1% agar and centrifuge to obtain a button of the biological material.
3. Cut the agar block and wash twice for 15 min with buffer containing sucrose to remove glutaraldehyde.
4. Postfix the agar cubes in 1% osmium tetroxide for 2 h and wash with Millonig's buffer twice for 15 min.
5. Dehydrate the specimen, clear in propylene oxide and embed in Epon 812.
6. Cut 1–2 μm thick section, mount on glass slides, stain by 0.5% toluidine blue and examine for proper orientation.
7. Cut the sections of 500–600 Å, mount onto uncoated copper grids, stain successively by 5% alcoholic uranyl acetate and 0.31% lead citrate.
8. Scan and photograph in EM.

3.3. Kinetic Characterization of Zinc Uptake into Renal BBMV (see Note 2)

3.3.1. Time-Course of Zinc Uptake

Zinc uptake into renal BBMV as a function of time is measured at 25°C by the Millipore rapid filtration technique as described earlier by Prasad et al. ***(18,19)***.

1. Incubate of reaction mixture, containing 20 μL of BBMV (100–125 μg protein) and 80 μL of uptake buffer at 25°C for different time intervals.
2. Terminate the uptake at the end of incubation by adding 4.0 mL of ice cold stop buffer.
3. Filter the reaction mixture immediately through nitrocellulose membranes (0.45 mm mesh) and rinse the filter three times with an additional 8.0 mL of stop buffer.
4. Count the retained radioactivity on the filters in Gamma Scintillation Counter and calculate specific activity after substracting the background / nonspecific counts.

3.3.2. Substrate Dependence of Zinc Uptake into Renal BBMV

In order to determine the kinetic constants, i.e., affinity constant (K_M) and maximal velocity (V_{max}) measure zinc uptake (steady state) measured at different substrate concentrations.

1. Incubate 20 μL BBMV (100–125 μg protein) with 80 mL of uptake buffer containing increasing concentration of Zn^{2+} (0.5 m*M* to 3.0 m*M*) and 1.0 μCi of ^{65}Zn.
2. Stop uptake and count the radioactivity as described in **Subheading 2.3.2.**

3.3.3. Zinc Uptake into the Renal BBMV Measured as a Function of pH

1. Measure the zinc uptake at 1 m*M* $^{65}Zn^{2+}$ concentration at 25°C in the presence of uptake buffer with different pH gradients.
2. Modify the pH of intra or extravesicular medium as follows:
   a. Inwardly Directed Proton Gradient:
      20 μL of BBMV (100–125 μg protein) prepared in reconstitution buffer (*see* **Subheading 2.2.**) were incubated with 80 mL of uptake buffer of varying pH (2.9–6.9).
   b. Outwardly Directed Proton Gradient:
      20 μL of BBMV (100–125 μg protein) reconstituted in buffers of varying pH (2.9–6.9) were then incubated with 80 μL of uptake buffer (pH 6.9) at 25°C. Prepare the uptake buffers of pH <6.0 with 15 m*M* (MES)-KOH. Measure zinc uptake as described in **Subheading 2.3.1.**

3.3.4. Effect of Temperature on Zinc Uptake into BBMV

1. Measure steady-state uptake of zinc at different temperature (10°C to 50°C in the presence of uptake buffer.
2. Construct an Arrhenius plot by plotting log *v VS* 1/*K*.
3. Calculate the energies of activation from the slope lines, using the Arrhenius equation.

$$Ea = \frac{2.303\ (log V_2 - log V_1) \times R \times T_2\ T_1}{(T_2 - T_1)}$$

Ea = Energies of activation (Cal/mol).
V_1 = Initial velocity at temp T_1.
V_2 = Initial velocity at temp T_2.
T_1 and T_2 = initial and final temp (°K).
R = gas constant (1.987 cal/mol).

3.3.5. Competition and Inhibition Studies

For the competition experiments, measure Zn^{2+} uptake into renal BBMV measured in the presence of 2 m*M* Cd^{2+} or 2 m*M* Ca^{2+} in the uptake buffer containing 1 m*M* Zn^{2+} and 1 μCi $^{65}Zn^{2+}$. To assess the involvement of sulfhydryal and carboxyl groups in zinc transport process, measure initial velocity zinc uptake in the presence of sulfhydryl group blocking agent (5 m*M* Iodoacetate) and carboxyl reacting compound (5 m*M* DCCD).

3.4. Purification of Major Zinc-Binding Protein from Renal Brush Border Membranes

Brush border membrane proteins are solubilized as described by Kumar and Prasad ***(26)***. Solubilized major zinc binding protein are purified using various

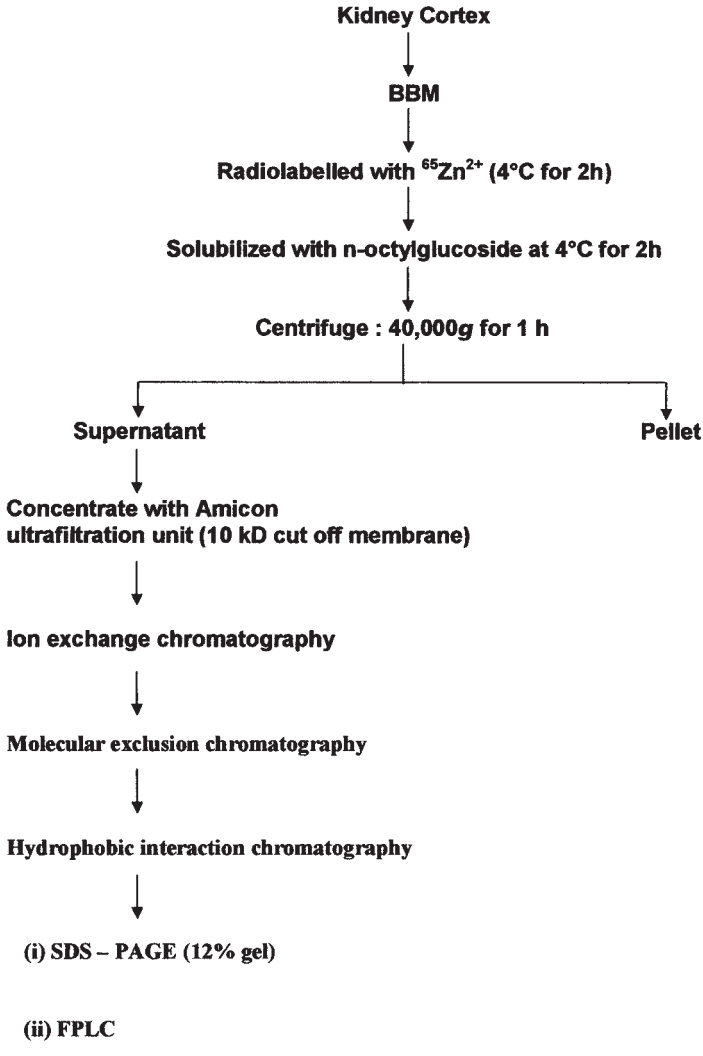

Fig 2. Scheme for Purification of MZnBP from renal BBM.

column chromatography techniques ***(26)***. The scheme for the purification of MZnBP is illustrated in **Fig. 2**.

*3.4.1. Solubilization of BBM Proteins (*see ***Note 3****)*

1. Incubate appropriate amount of BBM proteins (5.0 mg/mL) at 4°C for 1 h in a reaction mixture containing 1 m*M* Zn^{2+} and 10 μCi of $^{65}Zn^{2+}$ in solubilization buffer.
2. The solution is made 1% in *n*-octylglucoside (protein to detergent ratio 1 : 4 w/w) by the addition of detergent to a stirring solution at 4°C. Stir the reaction mixture for 1 h.

3. Separate solubilized proteins from insoluble material by centrifugation at 40,000*g* for 1 h.
4. Dialyze solubilized proteins for 10–12 h in a dialysis bag (10-kDa cutoff).
5. The solubilized proteins are subjected to following chromatography separation procedures.

3.4.2. Ion-Exchange Chromatograpy

1. Concentrate the dialyzed protein sample to 3 mL using Amicon ultrafiltration assembly with a 10-kDa cutoff filter.
2. Charge the proteins onto a DEAE-Sephadex A-25 column (12 cm × 1.2 cm), preequilibrated with 20 m*M* Tris-HCl buffer (pH 7.0) containing 1% lubrol PX.
3. Remove unbound proteins by washing the column with the same buffer (3× bed vol).
4. Elute the bound proteins with a linear ionic gradient (0–1.0 *M* NaCl). Collect 3.0-mL fractions at a flow rate of 12 mL/h.
5. Monitor each fraction for absorbance at 280 nm and measure $^{65}Zn^{2+}$ radioactivity as counts per minute in gamma scintillation counter.
6. Pool the fractions showing high zinc binding specific activity, desalt and concentrate by Amicon ultrafiltration using 10-kDa cutoff membrane.

3.4.3. Molecular Exclusion Chromatography

The concentrated pool of partially purified ZnBP from DEAE-Sephadex column is resolved on Sephadex G-100 column (85 × 1.4 cm)

1. Preequilibrate the column with 25 m*M* Tris-HCl buffer (pH 7.0) containing 1% lubrol PX.
2. Elute the proteins with the same buffer at a flow rate of 8 mL/h.
3. Collect 2.0-mL fractions and monitor for absorbance at 280 nm and count $^{65}Zn^{2+}$ as cpm.
4. Pool the fractions having high zinc binding specific activity and incubate with Bio-beads SM-2 at a concentration of 10 mg/mL to remove the detergent from the protein sample.
5. After filtration through a syringe filter, concentrate the protein sample to 1.0 mL in an Amicon ultrafiltration assembly using 10-kDa cutoff membrane.

3.4.4. Hydrophobic Interaction Chromatography

1. Load the concentrated protein sample from Sephadex G-100 column onto phenyl—Sepharose column (8 cm × 0.8 cm), preequilibrated with 25 m*M* Tris-HCl buffer (pH 7.0).
2. Remove unbound proteins by washing the column (3× bed vol with same buffer).
3. Elute bound proteins with a linear gradient of *n*-octylglucoside (0–2.0%).
4. Collect 800 μL fractions at a flow rate of 4.0 mL/h and monitor for absorbance at 280 nm, as well as for radioactivity (cpm).
5. Pool the fractions showing high zinc specific activity and remove the detergent by adding Bio-beads SM-2 (10 mg/mL).

6. Concentrate the sample in an Amicon ultrafiltration assembly using 10-kDa cutoff membrane filter.
7. Check the purity of the purified protein using SDS-PAGE and FPLC.

3.4.5. FPLC

1. Check the purity of purified protein on Sepharose column by charging in on FPLC using a Mono-Q HR 5/5 (Pharmacia) column.
2. Preequilibrate the column with preequilibration buffer.
3. Apply 100 μL sample (10 μg proteins) onto mono Q column and wash the column with preequilibration buffer.
4. Elute the bound protein with a linear gradient of chloride ions (0–500 m*M* NaCl).
5. Obtain the elution profile on chart recorder. The elution profile showed a single peak, suggesting that the protein has been purified upto homogeneity, which is further checked by SDS-PAGE.

3.5. PhysicoChemical Properties of Purified Major Zinc-Binding Protein

3.5.1. SDS-Polyacrylamide Gel Electrophoresis

1. Sample preparation: Dilute the sample with sample buffer (4:1) and boil at 95°C for 5 min.
2. Load the sample onto the wells of the gel (12% separating/4% stacking) with a Hamilton microsyringe.
3. Conduct the electrophoresis at constant current of 20 mA.

3.5.1.1. Silver Staining

1. Fix the gel in a fixing solution.
2. Discard the fixing solution, and add five volumes of ethanol : GAA : ddH_2O (20 : 5 : 75 by volume).
3. Discard the above solution and incubate the gel with 10 volumes of ddH_2O at room temperature for 10 min.
4. Add Former's solution and incubate at room temperature for 10 min. Discard the solution and wash the gels with ddH_2O until the yellow colour disappears.
5. Incubate the gel with $AgNO_3$ solution at room temperature for 30 min.
6. Wash the gel with ddH_2O three times.
7. Incubate the gel with freshly prepared developer solution till the bands start appearing and the required contrast is obtained.
8. Destaining is done with 5% GGA solution in ddH_2O.
9. Store the gel in ddH_2O at 4°C until it is photographed.

3.5.2. Metal Displacement Assay

The specificity of the purified protein for zinc is checked by metal displacement assay as described by Nelson et al. ***(18)***.

1. Incubate native protein (10 μg) with 10 μCi $^{65}Zn^{2+}$ at 4°C for 4 h.
2. Displace the bound ^{65}Zn in the presence of increasing concentrations of different metals (10 m*M*) (Zn^{2+}, Ca^{2+}, Cu^{2+}, and Cd^{2+}).
3. After 24 h, add 1 mL of a slurry of Chelax-100 (1 g/10 mL ddH_2O) to each sample.
4. Analyse the supernatant from the centrifuged samples for radioactivity.
5. Calculate bound and free Zn^{2+} concentration and calculate kinetic constant for Scatchard plot analysis.

3.5.3. UV Absorption Study

Further, the specificity of the protein is checked by UV absorption study as described earlier ***(28)***.

1. Incubate the sample of native protein (10 μg) with different metal ions (Zn^{2+}, Ca^{2+}, Cu^{2+}, and Cd^{2+}) and record the UV absorption spectra between 200 nm–400 nm on spectrophotometer.

3.5.4. Carbohydrate Content Estimation

Carbohydrate content in the purified ZnBP is determined by phenol-sulfuric acid microassay ***(29)***.

1. Incubate 10 mL of reagent A (5% w/v of distilled phenol).
2. Add 100 μL reagent B.
3. Add conc H_2SO_4 rapidly to the reaction mixture and leave undisturbed for 10 min. and shake vigorously and incubate at 37°C for 30 min.
4. Measure optical density at 490 nm. Use glucose (1–10 μg) as standard.

3.6. Functional Characterization

*3.6.1. Reconstitution of Solubilized BBM Proteins/Purified MZnBP into Proteoliposomes (*see ***Note 4****).*

Solubilized BBM proteins/purified, MZnBP are reconstituted into liposomes by the method as aforementioned ***(30,32)***.

1. Concentrate the protein sample to 1.0 mL in an Amicon ultrafiltration assembly using 10-kDa cutoff membrane and wash three times with KHT buffer.
2. Dissolve 36 mg of purified egg yolk phosphatidylcholine and 9 mg cholesterol under 100% nitrogen atmosphere in 1.0 mL of chloroform/methanol solution (chloroform to methanol ratio 2:1) in a round-bottomed flask.
3. Evaporate the solvent under reduced pressure with rotory evaporator.
4. Achieve complete removal of the solvent by further evaporation under high vacuum with a liquid nitrogen vapor trap.
5. Resuspend thin film of lipids in 10 mL of KHT buffer with a small magnetic stirring bar and transfer milky suspension into a test tube. Conduct all the earlier steps under nitrogen atmosphere.

6. Then freeze 1 mL of the sonicated lipids in a liquid nitrogen and then at room temperature, dialyze the proteins prior to the addition.
7. Dialyze the suspension after addition of 1.0 mL of KHT buffer at 4°C for 36 h against 6 × 1 L of KHT buffer.
8. After dialysis, dilute the suspension to 8.0 mL with reconstitution buffer and centrifuge for 10 min at 400*g* to remove any aggregated material.
9. Centrifuge the resulting supernatant for 1 h at 100,000*g* at 4°C to sediment the proteoliposomes.
10. Resuspend the pellet in 1.0 mL of reconstitution buffer and homogenize gently by aspirating the suspension into 1.0 mL syringe through a 26-gage needle.
11. Repeat this step three times.
12. Similarly, subject liposomes to the above procedure but without the addition of proteins and finally reconstitute in 1.0 mL of mannitol–HEPES buffer (pH 6.9).

3.6.2. Phase Contrast Microscopy

1. View the proteoliposomes under the phase contrast microscope in order to assess the structural integrity.
2. Dilute the proteoliposomes suitably and keep one drop on the cover slip.
3. Invert the cover slip onto the glass slide.
4. Keep the microscope on for 10 min before viewing the sample under light and dark field and photographed. Structural integrity of proteoliposomes is checked by phase contrast microscopy.

3.6.3. Zinc Efflux Study

Further, to check the structural integrity of the proteoliposomes, measure the efflux of Zn^{2+} in the presence of different divalent cations.

3.7. Immunohistochemical Localization of the MZnBP in Histological Section of Kidney

3.7.1. Raising Antisera

1. Emulsify the immunogen (100–150 μg) with an equal volume of FCA.
2. Inject emulsified antigen (100–150 μg) intradermaly on the shaven thigh of the rabbit.
3. Further, give booster dose of emulsified immunogen (50–60 μg in Freund's incomplete adjuvant) at seventh, fourteenth, twenty-first day.
4. Collect the blood in sterile test tube from the ear vein and separate the serum and store in aliquots of 100 μL at −70°C till further use.

3.7.2. Immunofluorescence Staining

Immunofluorescence staining is performed by the method described by Kumar and Prasad ***(26)***. In brief, frozen sections of tissues are fixed by keeping at −70°C for one day. The sections are incubated with the appropriate dilution of antisera (raised against the purified protein) at 37°C for 45 min. in a humidified

Table 3
Enzyme Activities of Homogenate and Brush Border Membrane Fraction of Rat Kideny Cortex

Fraction	Homogenate	Brush border membranes	Enrichment factor
Alkaline Phosphatase	0.085 ± 0.011	1.045 ± 0.095	12.20
Maltase	0.073 ± 0.013	0.905 ± 0.084	12.40
Na^+—K^+—ATPase	0.033 ± 0.002	0.009 ± 0.0008	0.27
(With Ouabain)	0.01 ± 0.003	0.002 ± 0.003	0.25

Enzyme activities are expressed as μmol/mg protein/min.

chamber and wash twice with PBS. Then, incubate the slides with FITC labeled anti-rabbit goat 1gG at 37°C for 45 min. After washing, mount the sections in 10% glycerine and view under the fluorescence microscope and photograph.

4. Notes

1. The BBMV employed in the present study fulfilled the criteria with respect to function, structural integrity and vesicularity. From measurements of marker enzymes (*see* **Table 3**), the renal membrane vesicles were found free from contaminations with basolateral membrane (ouabain-sensitive Na^+–K^+ ATPase) as revealed by enrichment factor less than 1.0 for this component. The quality of final renal brush border membrane vesicles preparations was evaluated by marker enzymes of brush border membrane. Specific activities of alkaline phosphatase and maltase were enriched about 12-fold when compared with the initial homogenate values. Electron microscopy of renal BBMV showed that most of the membrane formed smooth surface vesicles. The microvilli were well preserved. A dense material inside the vesicles was also seen (*see* **Fig. 3**). Preparation was free from the contamination of other organelles like nuclei, mitochondria, and microsomes.

   The ability of the vesicle preparation to support an inwardly directed sodium gradient provides strong evidence that the transient accumulation of D-glucose in own concentration gradient is characteristic of the model for sodium coupled–D glucose transport in BBMV.
2. Kinetic characterization of zinc uptake into renal BBMV suggests that this is a carrier mediated saturable phenomenon which follows Michaelis-Menten kinetics. Zinc uptake is pH dependent, temperature sensitive and $^-$COOH groups are essential for the transport of zinc ***(19,26,28)***. One of the requirements of carrier mediated transport of a metabolite is the competitive inhibition by its structural analogue. Zinc and cadmium share many physicochemical properties, and occur together in the *IInd* B-group in the periodic table and behave antagonistically to each other ***(33)***. The observed inhibition of the saturable zinc transport system in renal BBMV by cadmium is consistent with this hypothesis. It is noteworthy here that transport studies should preferably be conducted in freshly prepared membranes. pH of

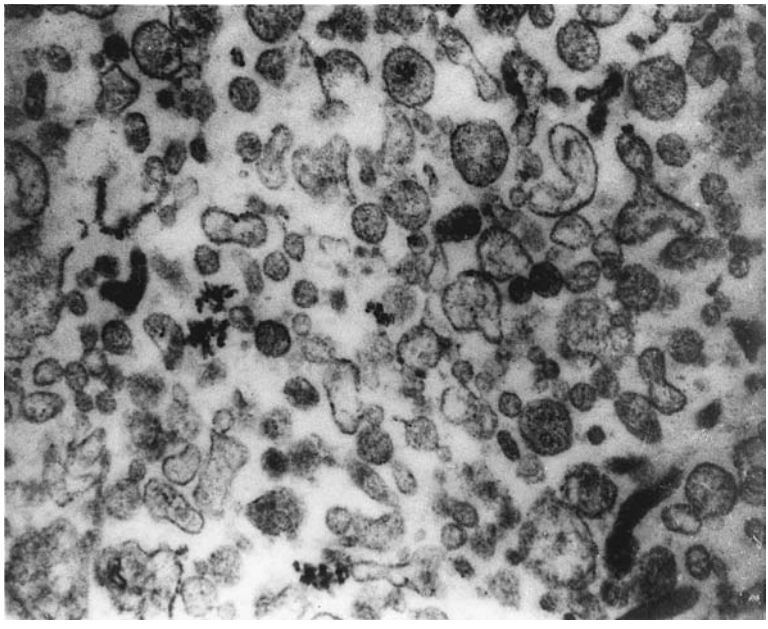

Fig. 3. Electron micrograph of BBMV. Vesicularity of BBMV was checked by electron microscopy (×45, 500).

uptake buffer is very critical. The pH of the uptake buffer should not exceed 7.0 because zinc salt will be precipitated.

All the steps including temperature, time and speed of centrifugation are critically important for obtaining good quality brush border membranes. There should be no delay during BBM preparation at any step, and especially at homogenization, to avoid proteolysis. The final pellet of BBM should be reconstituted by dispersion with the help of 1-mL tip autopipette, then passed through a 26-gage needle syringe for homogeneous preparation. Reconstituted BBMV should be kept at ice at least for 1 h before use for transport studies. BBM should be kept at −80°C before solubilization, purification, and functional reconstitution studies.

3. During the solubilization procedure, n-octylglucoside was best suited for the membrane proteins solubilization and purification. In the present study, approx 80% of the BBM proteins are solubilized by the n-octylglucoside treatment. Other have also used this detergent to solubilize renal phosphate and Na^+/D-glucose transporter and reported the similar yield after solubilization ***(34,35)***.

A 40-kDa MZnBP was purified from renal BBM to apparent homogeneity, using different chromatographic separation columns. Purification yield of MZnBP at different steps are mentioned in **Table 4**. About 80 μg protein was obtained from 46.75 mg of BBM proteins having 22-fold purification. Other zinc containing proteins which are 160-kDa and 225-kDa protein, respectively. Metals displacement and UV absorption assay revealed that the MZnBP is highly specific for zinc and

Table 4
Purification Fold of MZnBP at Different Steps of Purification

S. No.	Purification steps	Protein (mg)	Activity (cpm)	Sp. activity (cpm/mg protein)	% yield	Fold purification
I	BBM	46.75	–	–	–	–
II	Solubilized proteins	21.98	128275	5836	100	–
III	DEAE sephadex	2.39	124098	51924	10.87	9
IV	Sephadex G100	0.197	17670	89695	0.896	15
V	Phenyl sepharose	0.080	10328	129100	0.364	22

Fig. 4. Photomicrograph of proteoliposomes. Vesicularity of Proteoliposomes was checked by phase contrast microscopy. Proteoliposomes were viewed under dark field (400×).

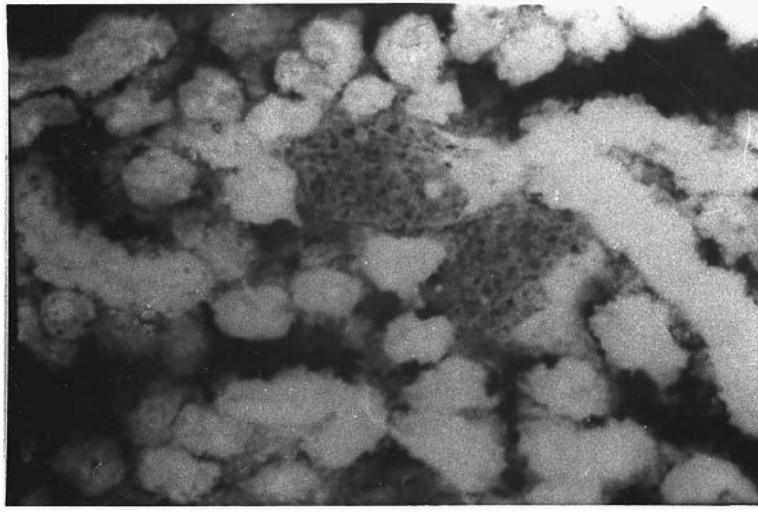

Fig. 5. Immunofluorescence localization of MZnBP in rat kidney (400×).

has high affinity and capacity for zinc. All purification steps should be carried out at 4°C.

During purification of transmembrane proteins, the detergent concentration should remain below the CMC to avoid inactivation aggregation of the membrane protein. Columns should be equilibrated with the buffer containing detergent below CMC. Detergent should also be included in the elution buffer.

4. A 40-kDa MZnBP from renal BBM was purified to homogeneity. Physical incorporation of the purified MZnBP was evident from SDS-PAGE as has been reported earlier for renal phosphate and Na^+/D-glucose cotransporter. Phase contrast microscopy showed that liposomes were unilamellar and boundaries were intact (*see* **Fig. 4**). Further Zn^{2+} efflux assay showed that around 85–90% of Zn^{2+} was retained in the preloaded proteoliposomes. Structural integrity and unilamellar nature of the liposomes are prerequisite for the use of proteoliposomes in the transport measurement studies.

   Kinetic characterization of zinc uptake into the proteoliposomes showed a slight increase in the K_M of the transporter for zinc when compared with the BBMV which could be due to the change in the microenvironment of the protein in proteoliposomes. However, there was approximately forty fold increase in V_{max}, which could be due to the presence of increased number of active transporters. Cadmium is a known competitive inhibitor of zinc uptake process in the renal BBMV. As expected zinc uptake in to the proteoliposomes was competitively inhibited by 2 m*M* Cd whereas Ca^{2+} did not show any effect on the zinc uptake. In our study, zinc transport into the reconstituted proteoliposomes was also temperature and pH dependent similar to zinc transport process into renal BBMV. Immunofluoroscence localization of the protein on the potential sites of zinc transport further confirms that the 40 kDa MZnBP is a zinc transporter (*see* **Fig. 5**).

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18

Isolation of Lipid Raft-Associated Proteolipids

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1. Introduction

1.1. Proteolipid (PL) Definition

The term proteolipid (PL) was coined more than 50 yr ago by Folch and Lees ***(1)*** to designate a complex protein fraction present in brain myelin that is coextracted with cell lipids using organic solvents. Subsequently, equivalent fractions were isolated from various sources, from bacteria to mammalian cells. This indicated that the existence of proteins with physical–chemical characteristics similar to those extracted from brain myelin is a general biochemical scenario. PLs are operationally defined as membrane proteins displaying unusual lipid-like properties that make them to partition into lipophilic solvents ***(2)***. Although no clear-cut distinction was originally made between PLs and lipoproteins ***(1)***, today it is evident that proteolipids constitute a different protein group from that of lipoproteins, as the latter term designates water-soluble complexes composed of proteins and lipids (e.g., high-[HDL] and low-[LDL] density lipoproteins).

The PL group includes proteins of very different structures and functions. Some of the members of this group possess a lipid moiety covalently attached to the protein contributing to their solubility in lipophilic solvents. However, this is not a requisite for being a PL as other members of the group do not have such a modification. The archetypal PL is the proteolipid protein (PLP), which is a major component of myelin membranes. PLP contains four transmembrane domains and has covalently linked fatty acids ***(3)***. Between the nonmyelin proteolipids, the 16-kDa subunit (c subunit) of eukaryotic vacuolar adenosine triphosphate (ATP) synthase, also called H^+-ATPase or V-ATPase ***(4)***, is one of

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the best characterized. This protein arose from duplication of the 8-kDa PL component of the ATP synthase (F-ATPase) of chloroplasts, mitochondria, and bacteria ***(5)***. The γ subunit of V-ATPase ***(6)*** and the human surfactant PL ***(7)*** are examples of PLPs containing a single membrane-spanning domain.

1.2. Membrane Microdomains ("Lipid Rafts") in Cellular Membranes

Cellular membranes were envisaged for long time as being disordered uniform bilayers in which lipids moved freely and randomly by lateral diffusion. A new model proposes the existence in biological membranes of lipid microdomains, or rafts, that have a high sphingolipid and cholesterol content. Unlike the loosely packed, disordered phospholipids present in the bulk of membranes, raft lipids are organized in a tightly packed, liquid-ordered manner ***(8)***. A current model of raft structure proposes that sphingolipids, which contain a sphingosine chain and a long, largely saturated, fatty acyl chain, are packed together with cholesterol in small membrane structures ***(9)***. The tight packing of lipids in the rafts confers resistance to solubilization by nonionic detergents at low temperatures, which allows their isolation as a detergent-insoluble membrane fraction using centrifugation to equilibrium in sucrose density gradients ***(10)***. The peculiar organization of the rafts restricts the access of proteins in such a way that only proteins attached to the membrane by a lipid anchor, such as glycosylphosphatidylinositol (GPI)-anchored proteins or acylated cytosolic proteins, and certain integral membrane proteins can reside in rafts, whereas the majority of integral membrane proteins are excluded.

1.3. The MAL Protein

1.3.1. MAL Is a PL

MAL cDNA was originally cloned during a search for genes that are differentially expressed during T-cell ontogeny ***(11)***. In addition, MAL was also shown to be expressed in polarized epithelial cell lines ***(12)*** and myelin-forming cells ***(13)***. The encoded protein, named MAL, is a nonglycosylated protein of 17 kDa containing four putative membrane-spanning domains. The MAL protein did not present any significant overall homology with any protein sequence deposited in the GenBank at the time its cDNA was cloned. Despite this, the hydrophobicity profile of MAL was remarkably similar to that of the *c*-PL subunit of the V-ATPase ***(14)***. This prompted us to demonstrate MAL's first unusual biochemical feature, whereby it partitions in chloroform:methanol mixtures or *n*-butanol ***(12)***. These lipid-like properties allowed us to include MAL in the proteolipid group.

The advance in the sequencing of encoded sequence tags (ESTs) in the last few years has led to the identification of novel proteins with overall sequence

identity with MAL. MAL is, therefore, the founder member of an extended family of proteins, the MAL proteolipid family, which all have structural and biochemical similarities ***(14)***.

1.3.2. MAL Associates With Lipid Rafts

A second unusual biochemical feature of MAL is its selective residence in lipid rafts in all the cell types in which is expressed ***(12,13,15)***. This property is probably related to the intrinsic lipid-like characteristics of MAL that make MAL to be compatible with raft membranes. In mammalian cells, the incorporation of MAL into rafts requires a pre-Golgi sorting event mediated by a sequence in its carboxyl terminal end ***(16,17)***. In the absence of this sequence, MAL is misrouted to standard membranes, which, in contrast to raft membranes, are solubilized by nonionic detergents. BENE, the second member of the MAL family to have been characterized to date, behaves also as a PL and resides exclusively in raft membranes ***(18)***.

1.3.3. Function of MAL

Recruitment of specific proteins into rafts was initially proposed to explain the segregation and transport of apical proteins during biosynthetic transport in polarized epithelial cells ***(19)***, and was subsequently suggested as being a general mechanism for protein recruitment in a variety of processes including membrane trafficking and signaling ***(8)***. To organize raft membranes to make them functional, the existence of specialized protein machinery was postulated. MAL has recently been demonstrated as being a component of the integral protein machinery for raft-mediated apical transport of polarized epithelial Madin–Darby canine kidney (MDCK) cells, which are a paradigm of polarized epithelial cells. The role of MAL in apical transport is essential as depletion of endogenous MAL severely reduces apical transport of specific exogenous and endogenous integral membrane proteins, proteins attached to the membrane by a GPI moiety and secretory proteins ***(20–23)***.

2. Materials

1. Phosphate-buffered saline (PBS): .137 m*M* NaCl, 2.70 m*M* KCl, 4 m*M* Na_2HPO_4, 1.76 m*M* KH_2PO_4, pH 7.2.
2. Triton X-100; sucrose; Coomassie blue (Sigma Chemical Co., St. Louis, MO).
3. Ultra-Clear centrifuge tubes (Beckman Instruments, Palo Alto, CA).
4. Laemmli gels ***(24)*** for electrophoresis prepared using 10–15% polyacrylamide.
5. Gel stain: 0.1% Coomassie blue in 10% acetic acid, 50% methanol, 40% H_2O.
6. Gel destain: 10% acetic acid, 50% methanol, 40% H_2O.
7. Anticaveolin antibodies (Transduction Labs, Lexington, KY).
8. Antitransferrin receptor antibodies (Zymed, San Francisco, CA).

9. Immobilon™-P membranes (Millipore, Bedford, MA).
10. Antihuman MAL mAb 6D9 ***(12)***.
11. Anti-IgG antibodies coupled to horseradish peroxidase (Pierce, Rockford, IL).
12. Enhanced chemiluminescence kit (ECL, Amersham Biosciences, Uppsala, Sweden).
13. Reagent grade *n*-butanol, chloroform and methanol (Merck, Darmsdatd, Germany).
14. Sephadex LH-20 beads (Amersham Biosciences).
15. 3M chromatograpy paper (Whatman, Maidstone, England).
16. Buffer A: 25 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* ethylenediaminetetraacetic acid (EDTA).

3. Methods

The procedure for raft-associated PLs purification is based on the two most specific biochemical features of MAL: exclusive presence in lipid rafts and solubility in lipophilic solvents. These features are exploited for a two-step purification procedure, which combines the separation of lipid rafts from cultured cells and the isolation of the proteolipid fraction from the rafts (*see* **Fig. 1**). The procedure used for MAL purification described later, which is based on the lipid-like properties and raft association of MAL, is also likely to be of use for the isolation of BENE ***(18)***, as well as for other members of the MAL family.

3.1. Cell Lysis

1. For mammalian cells growing attached to plastic dishes, four 100-mm diameter confluent dishes are prepared as starting biological material (*see* **Notes 1** and **2**).
2. The cells are rinsed with phosphate-buffered saline (PBS) and lysed for 20 min in 1 mL Buffer A plus 1% Triton X-100 at 4°C (*see* **Note 3**).
3. The lysate is scraped from the dishes with a cell lifter, and the dishes are then rinsed with 1 mL of the same buffer at 4°C.
4. The lysate is homogenized by passing the sample through a 22-gage needle.

3.2. Sucrose Density Gradient Preparation

1. The cell extract is brought to 40% sucrose in a final volume of 4 mL using 80% sucrose in Buffer A.
2. The sample is placed at the bottom of an ultracentrifuge tube and is sequentially overlaid with 6 mL of 30% sucrose and 2 mL of 5% sucrose in Buffer A.

3.3. Isolation of the Raft Fraction

1. Gradients are centrifuged for 18 h at 39,000 rpm at 4°C in a Beckman SW41 rotor.
2. The opalescent floating band, which consists of the Triton X-100 raft fraction, at the 5–30% sucrose interface is collected in approx 1 mL.
3. The 40% sucrose layer on the bottom of the tube (4 mL), which contains the Triton X-100 soluble material, is also harvested.
4. Equivalent aliquots from the starting material and the raft and soluble fractions are analyzed by immunoblot with anti-MAL mAb 6D9 and by Coomassie blue stain-

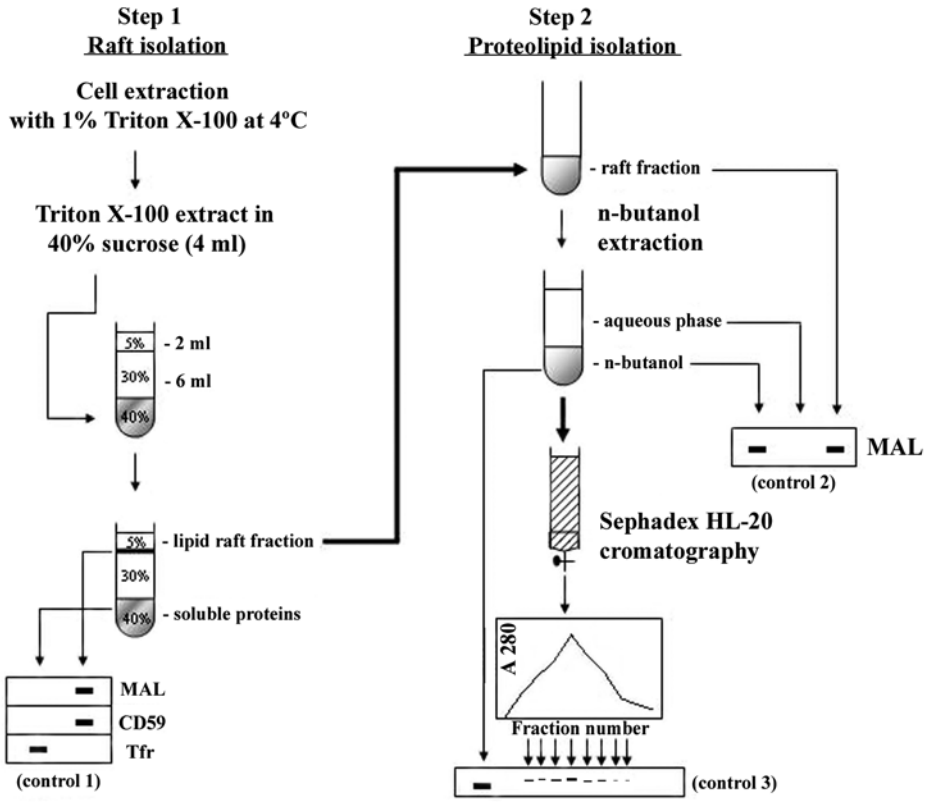

Fig. 1. Schematic representation of the two-step procedure used to isolate the MAL proteolipid from cell cultures.

ing to determine the recovery of MAL in the raft fraction and the protein content, respectively (*see* **Notes 4** and **5**).

3.4. Isolation of the PL Fraction

1. The lipid raft fraction is solubilized with 1% Triton X-100 at 37°C for 30 min (*see* **Note 6**).
2. The solubilized lipid raft fraction is extracted with an identical volume of *n*-butanol for 15 min at room temperature, shaken vigorously, and centrifuged at low speed (*see* **Note 7**).
3. The aqueous phase is withdrawn and reextracted with an equal volume of *n*-butanol.
4. The organic phases resulting from the two extractions are filtrated through a 3M filter paper and pooled.
5. An aliquot of the organic solvent pool is dried and resuspended in electrophoresis loading buffer. Equivalent aliquots from the raft fraction and the organic solvent extract are subsequently analyzed (*see* **Note 8**).

3.5. Lipid Removal

1. To delipidate the sample, the organic phase is subjected to molecular filtration chromatography using Sephadex LH-20 beads equilibrated in *n*-butanol. The beads are packed in a siliconized Pasteur pipet and *n*-butanol is used as eluent. The elution of protein is monitored by measuring the absorbance at 280 nm of the effluent (*see* **Note 9**).
2. Aliquots from the different fractions are dried under nitrogen and resuspended in electrophoresis loading buffer. Equivalent aliquots from the starting organic solvent extract and the LH-20 chromatography fractions are analyzed by immunoblot with anti-MAL mAb 6D9 and stained with Coomassie blue to determine the recovery of MAL and the total protein content, respectively, in the different fractions.

4. Notes

1. A similar procedure can be followed for cells growing in suspension, such as T-cell lines. In this case, 100 mL of a cell culture grown at 2×10^6 cells/mL are centrifuged at low speed to collect the cells and then washed twice with PBS. Cells are subsequently extracted as described for cells grown attached to a plastic substrate.
2. The number and size of the cells used are critical. If suboptimal number of cells are used, rafts are solubilized, whereas an excess of cells in the contaminates the raft fractions with otherwise soluble membranes that are not completely solubilized. In our experience, 2–4 confluent 100-mm diameter Petri dishes give a reliable separation of soluble and raft fractions.
3. Although the definition of the raft fraction was originally restricted to membranes resistant to extraction with 1% Triton X-100 at 4°C (1% Triton X-100 rafts), this criterion has since been relaxed. It is now acceptable to use lower concentrations of Triton X-100 (e.g., 0.5%, 0.2%) and the rafts are referred to as 0.5% Triton X-100 or 0.2% Triton X-100 rafts. Similarly, detergents other that Triton X-100 (e.g., Brij series, CHAPS, Lubrol) can be used and the rafts are referred to as Brij, CHAPS, or Lubrol rafts.
4. It is useful to analyze the fractionation of control proteins as a means of assuring that the separation procedure works properly. Caveolin or GPI-anchored proteins (e.g., Thy-1, CD59, CD55) can be used as raft markers. The transferrin receptor (CD71) can be used as a marker for the soluble fraction.
5. Raft isolation gives routinely a 10^3–10^4-fold enrichment in the MAL protein as determined by comparison of the MAL signal obtained by immunoblot analysis with anti-MAL mAb 6D9 of the initial lysate and the final raft fraction.
6. This treatment solubilizes lipid rafts (*10*). The use of solubilized rafts is essential to obtain a good efficiency in the subsequent extraction of PLs with organic solvents.
7. A 2:1 (v:v) chloroform:methanol mixture (15 vol) can be used in substitution of *n*-butanol for the isolation of proteolipids from the raft fraction. In this case, 0.2–0.3 vol water are added to the extract and mixed for 30 min at 4°C to promote phase separation.
8. The recovery of MAL in the organic fraction is nearly 100% of that in the initial

raft fraction, as determined by immunoblot analysis with anti-MAL mAb 6D9, whereas >90–95% of the raft's total protein content remains in the aqueous phase, as measured by Coomassie blue staining.

9. Alternatively, instead of using LH-20 chromatography, to get rid of the lipids from the organic solvent extract we first dry the extract out and then resuspend the sample in a small volume (100 μL) of *n*-butanol. After centrifugation for 1 min in a microfuge, most of the lipids are present in the supernatant, whereas the pellet contains the proteolipids in an insoluble form.

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Purification of Membrane-Bound Catechol-*O*-Methyltransferase

Maria João Bonifácio and Patrício Soares-da-Silva

1. Introduction

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) was first detected in rat liver extracts ***(1)*** and since then, COMT has been found in plants, yeast, invertebrates, and vertebrates. In mammals, the highest COMT activities are encountered in liver, kidney, and gut wall ***(2,3)***. The general physiological function of COMT is the inactivation of biologically active or toxic catechols. The enzyme catalyzes the transference of a methyl group from S-adenosyl-L-methionine (SAM) to a hydroxyl group on a catechol substrate in the presence of magnesium and the reaction products are the *O*-methylated catechol and S-adenosyl-L-homocysteine ***(1)***.

COMT occurs in two distinct forms, a rough-endoplasmic reticulum membrane-bound form (MB-COMT), and a soluble form (S-COMT) present in the cytoplasm, which is the most abundant form ***(4)***. In humans and rats, a single gene encodes both forms of COMT using two separate promoters and translational regulation ***(5)***. S- and MB-COMT have identical primary structures, but the membrane-bound form has an additional short peptide (43 amino acids in rat and 50 amino acids in humans) in its amino terminal containing the membrane anchor region ***(4)*** MB-COMT is an integral membrane protein with the catalytic portion of the enzyme oriented towards the cytoplasmic side of the membrane ***(6)***. The kinetic behavior of MB-COMT differs from the S-COMT, in that MB-COMT has a 100-fold higher affinity for catechol substrates, with the exception of catechol estrogens ***(7,8)***.

We were interested in studying the affinity of MB-COMT upon removal from its native environment. In this chapter, we summarize the work we have

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performed with MB-COMT. We describe the isolation of the microsomal fraction of rat liver homogenates, the solubilization of this fraction and the partial purification of a fraction containing MB-COMT. We also describe the kinetic analyses performed to study the membrane-bound enzyme behavior.

2. Materials

1. 0.9 % (w/v) sodium chloride (NaCl).
2. Sodium pentobarbital 30 mg/ml. Prepare fresh in 0.9 % (w/v) NaCl.
3. Surgical equipment (scissors, hemostats, forceps, 21-gage needles)
4. Buffers: 5 m*M* sodium phosphate buffer (pH 7.8) and 20 m*M* Tris-HCl, pH 7.8.
5. Homogeneizer (Diax, Heidolph).
6. Ultracentrifuge Beckman.
7. 10% Triton X-100 in 20 m*M* Tris-HCl, pH 7.8.
8. Buffer A: 0.5% Triton X-100 in 20 m*M* Tris-HCl, pH 7.8.
9. Buffer B: 1 *M* NaCl 0.5% Triton X-100 in 20 m*M* Tris-HCl, pH 7.8.
10. Resource Q column of 6 mL (Amersham Biosciences).
11. Fast protein liquid chromatography (FPLC) with ultraviolet (UV) and conductivity monitors (Amersham Biosciences).
12. Protein quantification system—the Bio-Rad protein assay (Bio-Rad) was used according to the manufacturer's instructions. We also use a standard curve (50–250 μg/mL) of bovine serum albumin (BSA) protein (Bio-Rad).
13. Enzyme assay buffer twofold concentrated: 100 μ*M* $MgCl_2$, 1 m*M* ethylene glycol *bis* (d-aminoethyl ether)-*N,N,N′, N′*,-tetraacetic acid (EGTA), 100 μ*M* pargyline, 500 μ*M* SAM in 5 m*M* phosphate buffer pH 7.8. Prepare fresh and keep in the dark because SAM is light sensitive. Store in ice until use.
14. Epinephrine in enzyme assay buffer (0.5–1000 μ*M*). Again, prepare fresh and keep in the dark.
15. 2 *M* perchloric acid (PCA, corrosive solution so care must be taken when preparing and using it).
16. 0.22 μm pore size Spin-X filter tubes (Costar).
17. High-pressure liquid chromatography (HPLC) system (Gilson) with a stainless steel 5-μm ODS column (Biophase; Bioanalytical Systems) of 25 cm length. Detection system consisted of a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141).
18. Metanephrine in 0.2 *M* PCA. A stock solution (1 mg/mL) kept in the dark and at 4°C is stable for 1 mo. From this stock, we prepared each week the dilutions to use (100 ng/mL).
19. Mobile phase: 0.1 m*M* citric acid, 0.5 m*M* sodium octyl sulfate, 0.1 *M* sodium acetate, 0.17 m*M* ethylene diamine tetraacetic acid (EDTA), 1 m*M* dibutylamine, and 10% methanol, pH 3.5 with concentrated perchloric acid. The mobile phase was filtered through a 0.22-μ*M* filter and subsequently degassed. It has a stability of 1 mo.

3. Methods

3.1 Separation of the Cytosolic From Microsomal Fraction

Liver was the organ chosen to isolate the MB-COMT because it is where the highest activities of COMT are found, although by Western blot analysis only 7% of total COMT is MB-COMT ***(4)***. The separation of the cytosolic fraction (that contains S-COMT) from the microsomal fraction (containing MB-COMT) was performed by centrifugation ***(9)***.

1. Anesthetize rat with sodium pentobarbital (60 mg/kg intraperitoneally) and perfuse animal through the left ventricle with 20 mL 0.9 % sodium chloride (*see* **Notes 1** and **2**).
2. Remove liver, wash in 0.9% sodium chloride, clean of connective tissue, and mince the tissue. Place in 10-mL ice-cold 5-m*M* phosphate buffer (pH 7.8).
3. Homogenize minced livers by three bursts of 30 with the Diax homogenizer set at position 5 and transfer the homogenate to centrifuge tubes (*see* **Note 3**).
4. Centrifuge at 15,000*g* for 20 min at 4°C. Supernatant is then centrifuged at 100,000*g* for 1 h at 4°C (*see* **Note 4**).
5. Store the supernatant if determination of S-COMT activity is to be made. Wash the pellet twice (100,000*g* for 1 h at 4°C) with 10 mL 20 m*M* Tris. Resuspend the final pellet in 2–4 mL 20 m*M* Tris.
6. Determine total protein concentration in the membrane suspension.

3.2 Solubilization of the Membrane Fraction Containing MB-COMT Activity

The use of detergents is required to solubilize and extract membrane proteins from membranes. The detergent chosen was the non ionic detergent Triton X-100. Its monomer has a relative molecular mass of 625 and the critical micellar concentration is 0.3 m*M*. It is widely used for protein solubilization and it has also been used to solubilize MB-COMT ***(10,11)***. Preliminary experiments performed with different concentrations of Triton X-100, showed that detergent concentrations up to 1% did not change significantly the enzyme activity of either S- or MB-COMT. Furthermore, the presence of NaCl did not improve MB-COMT solubilization as expected, because the latter is an integral membrane protein (*see* **Note 5**).

1. Add to the membrane suspension 1/10 vol 10% Triton X-100 in 20 m*M* Tris (*see* **Note 6**).
2. Incubate for 30 min on ice and centrifuge at 100,000*g* for 30 min at 4°C to remove unsolubilized material.
3. The resulting supernatant that constitutes the solubilized membrane fraction should be composed by mixed micelles of lipid-detergent, lipid-protein-detergent, and protein-detergent. Store the pellet to evaluate residual enzyme activity.

3.3 Partial Purification of MB-COMT

To ascertain the effect of lipid removal from the solubilized membrane preparation we further purified the solubilized MB-COMT by anion-exchange chromatography. For that purpose, we have used a Resource Q column, which is prepacked with Source 15Q a media that combines high-speed, high-capacity with high resolution.

1. Equilibrate a Resource Q column (6 mL) connected to an FPLC system with Buffer A.
2. Dilute the solubilized membrane preparation with buffer without Triton X-100 to obtain a final concentration of detergent of 0.5% (*see* **Note 7**). Apply the solubilized membrane fraction onto the column using a Superloop of 10 mL and run the chromatography using the following conditions: flow of 2.0 mL/min until the sample is completely loaded and 4.0 mL/min afterwards. After loading the sample, wash unbound material with 30 mL buffer A and start gradient with buffer B 0–50% in 120 mL and 50–100% in 10 mL. Collect fractions of 2 mL during gradient application. Analyze collected fractions for the presence of the enzyme by determining the enzyme activity or by performing Western blotting analysis using an anti-COMT specific antibody.

In **Fig. 1,** it is shown a typical chromatogram obtained with Resource Q. The inset shows a western blotting analysis performed according to standard protocols ***(12)***. The antibody used was a rabbit polyclonal raised against pure recombinant rat COMT ***(13)*** that was further purified by affinity chromatography using an NHS-activated HiTrap column (Amersham Biosciences) to which recombinant rat COMT had been coupled.

3.4 Enzymatic Analyses

COMT activity was evaluated by measuring the ability of the enzyme to catalyze the methylation of epinephrine to metanephrine in the presence of magnesium and SAM ***(14–16)*** MB-COMT has a 100-fold higher affinity for catechol substrates than S-COMT, thus we performed concentration response curves to epinephrine and determined the K_m for the different preparations: the membrane-bound fraction, the solubilized membrane-bound fraction, and the partial purified MB-COMT.

1. Preincubate 100 μL of each of the enzyme preparations (membrane-bound, solubilized membrane-bound, and partial purified membrane-bound) with 80 μL of twofold concentrated enzyme assay buffer for 20 min at 37°C in a water bath with agitation.
2. Initiate the reaction by adding 20 μL epinephrine solutions (10-fold concentrated) of increasing concentrations.
3. Incubate for 5 min at 37°C in water bath with agitation and in the dark. Stop reaction with the addition of 50 μL 2 *M* PCA to precipitate proteins and place samples at 4°C for a minimum period of 2 h.

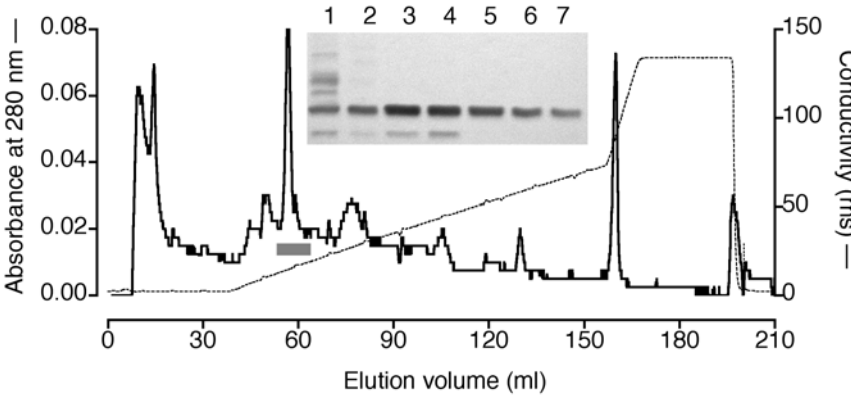

Fig.1. Elution of MB-COMT from a Resource Q column at pH 7.8 by an NaCl gradient. The full line represents the absorbance read at 280 nm and the dashed line represents the conductivity read on-line. The gray bar indicates the fractions positive for COMT activity. The inset represents an analysis by western blot of the MB-COMT microsomal fraction ***(1)***, the solubilized MB-COMT fraction ***(2)***, and the Resource Q fractions positive for COMT activity ***(3–7)***.

4. Centrifuge samples at 200*g* for 3 min in a laboratory microfuge and filter supernatants through 0.22 μ*M* SpinX filters. Inject filtered samples onto HPLC and measure metanephrine formed by electrochemical detection.

The results obtained are presented in **Fig 2**. The affinity of the membrane-bound fraction (K_m = 2.5 μ*M*) decreased with the solubilization (K_m = 9.3 μ*M*) and with the partial purification by ion exchange (K_m = 252 μ*M*). The latter value is not different from the K_m obtained for S-COMT (K_m = 257 μ*M*) ***(17)***. It appears, therefore, that the removal of the enzyme from its native environment changes its kinetic properties to the point of conferring it the same affinity of the soluble enzyme, which might have important implications for structural studies with MB-COMT.

4. Notes

All procedures, except the chromatography, are carried on ice with ice-cold solutions.

1. All animal manipulations have to be carried out according to existent legislation.
2. The purpose of perfusing organs with saline solution was to diminish contamination of hepatic COMT with erythrocyte COMT.
3. In initial experiments a teflon homogeneizer (Heidolph) was used; however, no significant differences were observed between the two homogenization procedures. Homogenization with Diax is quicker, thus, if one wants to process a higher number of tissues or is time limited, is the advised one.

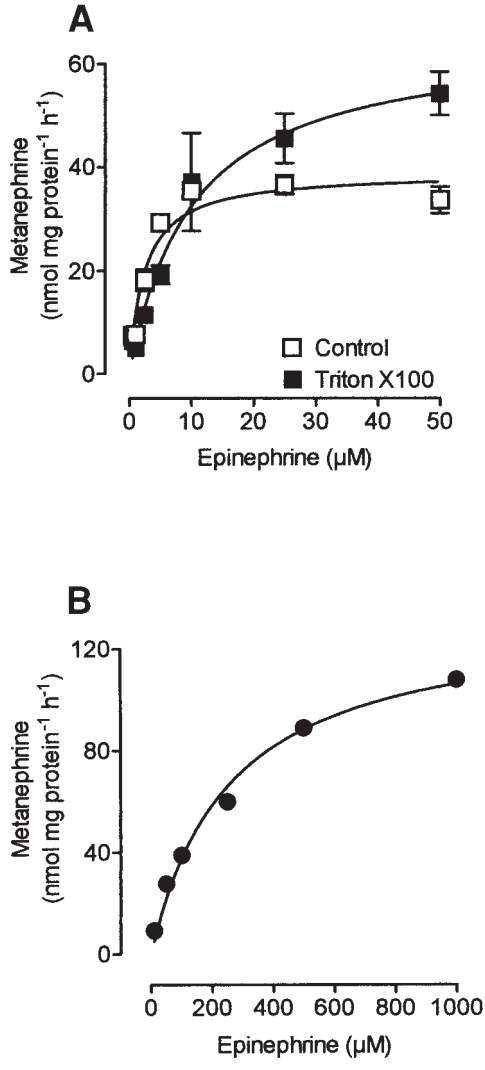

Fig. 2. *O*-methylation of epinephrine by **(A)** MB-COMT from rat liver microsomes (Control, open squares), solubilized microsomes (Triton X-100, closed squares) and **(B)** after Resource Q (closed circles). COMT activity is expressed as the rate of metanephrine formation in nmol mg protein^{-1} h^{-1}.

4. The supernatant of the 15,000*g* centrifugation has a fluffy white layer that is very difficult to avoid. We prefer to lose some supernatant, but have it as clean as possible.
5. Experiments where the solubilization conditions were tested showed that the presence of salt (0.1–0.75 *M* NaCl) did not improve the recovery of COMT activity in supernatants although higher salt concentrations extract more protein. Higher COMT activity was obtained with 0.5% Triton X-100, but some residual activity was observed in the pellet of unsolubilized material.
6. Several ratios detergent:protein were used, from 0.6:1 to 20:1. However, we prefer to use a total protein concentration of 5–10 mg/mL for solubilization with 10% Triton X-100 (detergent protein ration (20–10:1).
7. We have tried to use 1% Triton X-100 during the ion exchange chromatography, however, at this concentration the protein did not bind the matrix. At 0.5% Triton X-100 it was possible to perform the separations.

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Purification and Characterization of Transporter Proteins from Human Erythrocyte Membrane

Da-Neng Wang, M. Joanne Lemieux, and Jonathan M. Boulter

1. Introduction

Functions and biochemical properties of several membrane transporter proteins from human erythrocyte, in particular, the glucose transporter (Glut1) and anion exchanger (AE1, also called Band 3) have been extensively characterized. Glut1 is a member of the mammalian facilitative glucose transporter family Glut1-13 ***(1,2)***. The 50-kDa integral membrane protein is expressed in all human cells, and is particularly abundant in human erythrocytes, fibroblasts and endothelial cells. In erythrocytes it is responsible for the uptake of glucose—the cell's major energy source. Glut1 consists of 492 amino acids and is *N*-glycosylated on residue Asn45 in a highly heterogeneous manner. The protein, which functions as a monomer, is predicted to span the membrane 12 times in the form of α-helices ***(1)***.

A member of the anion exchanger family, AE1 is a 95-kDa integral membrane protein with multiple functions ***(3)***. It is responsible for the electroneutral exchange of HCO_3^- and Cl^- across the erythrocyte membrane, thereby facilitating CO_2 transport by the blood. The AE1 protein consists of two structurally distinct domains, an amino-terminal cytosolic domain (residues 1–360) and a carboxyl-terminal membrane domain (residues 361–911) ***(4)***. The cytosolic domain is approx 43 kDa, and provides binding sites for hemoglobin and cytoskeletal proteins. On the other hand, the 52-kDa membrane domain (AE1MD), which is glycosylated, is solely responsible for the protein's ion transport activity, and retains functionality following enzymatic removal of the cytosolic domain.

Easy access to human blood and their abundance in the erythrocyte mem-

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Membrane Protein Protocols: Expression, Purification, and Characterization
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brane have made Glut1 and AE1MD prototypes of integral membrane proteins for biochemical and biophysical studies *(5,6)*. In this work, we present two purification protocols for Glut1 and AE1MD. The protocol for Glut1, developed in our laboratory, introduces a positive chromatography step. As a result, less lipid is copurified whilst the protein still retains ability to bind to its inhibitor cytochalasin B in detergent solution *(7)*. The protocol for purifying AE1MD is derived from a procedure by Casey et al. *(8)*, to which an additional membrane stripping and chromatography step is introduced. The first Q anion exchange column is followed by deglycosylation of the protein, and the second Q column removes glycosidase and detached sugars and delipidates the protein *(9)*. Stability and monodispersity of purified Glut1 and AE1MD are subsequently analyzed under a wide range of conditions by analytical size-exclusion high-pressure liquid Chromatography (HPLC). AE1MD generated by our procedure yields three-dimensional crystals, diffracting to 14 Å resolution *(9)*.

2. Materials

2.1. Equipment

1. Preparative centrifuge: Sorvell RC 5B centrifuge with GS-3 and SA-600 rotors (Du Pont, Wilmington, DE).
2. Ultracentrifuge: Beckman XL-90 ultracentrifuge with Ti45 and Ti70.1 rotors (Beckman, Palo Alto, CA).
3. FPLC system: controller LCC-501 PLUS, peristaltic pump P-1, pumps P-500, valves MV-7 and MV-8, detector UV-M II, fraction collector FRAC-100, and recorder REC-102 (Amersham Pharmacia, Piscataway, NJ). The FPLC system is kept at 4°C in an Isotemp incubator (Fisher Scientific, Pittsburgh, PA).
4. Preparative ion-exchange chromatography columns: HiTrap Q 1 mL and 5 mL columns (Amersham Pharmacia).
5. Vertical electrophoresis system: Mini-Protean II xi vertical electrophoresis cell and gel casting system, and PowerPac 1000 or 3000 power supply (Bio-Rad, Hercules, CA).
6. HPLC system: Waters solvent delivery system (controller 600 and pump 626), controlled by the Millennium software on a PC, and photodiode array detector Waters 996 (Waters, Milford, MA).
7. Spectrophotometer: Agilent UV-Visible spectrophotometer 8453 (Agilent Technologies, Palo Alto, CA).
8. Analytical size-exclusion chromatography columns: Shodex KW803 and KW804 columns (Showa Denko, Tokyo, Japan).
9. Transmission electron microscope: CM12 electron microscope with a single-tilt, room-temperature specimen holder (Philips, Eindhoven, The Netherlands).

2.2. Reagents

1. Detergents, solubilization grade or higher (Anatrace, Maumee, OH).
2. Phospholipids (Avanti Polar Lipids, Alabaster, AL).
3. Peptide: *N*-glycosidase F (PNGase F) (New England BioLabs, Beverly, MA).
4. Trypsin (Worthington Chemicals, Toronto, Canada).
5. Micro-BCA Protein Assay Kit (Pierce, Rockford, IL).
6. Electron microscopy copper grids (Ted Pella, Redding, CA).
7. Filter paper type #41 (Whatman, Clifton, NJ).
8. All other reagents are from Sigma (St. Louis, MO) and are of analytical grade or higher.
9. All purification steps are carried out at 4°C unless specified otherwise. Purified proteins samples are stored at 4°C.

2.3. Buffers for Glut1 Purification

1. Phosphate-buffered Saline (PBS) buffer: 5 m*M* Na_2HPO_4, pH 7.4, and 150 m*M* NaCl.
2. Haemolysis buffer: 0.1 *M* phosphate buffer, pH 7.4, 0.5 m*M* ethylene diamine tetraaretic acid (EDTA), and 1 m*M* phenylmethyl sulfonyl fluoride (PMSF).
3. Stripping buffer: 15 m*M* NaOH, pH 12, 2 m*M* EDTA, and 0.2 m*M* PMSF.
4. Solubilization buffer: 20 m*M* Bis-Tris, pH 7.0, 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* PMSF, 10% glycerol, and 2% decylmaltoside (DM).
5. Loading buffer: 10 m*M* Bis-Tris, 0.5 m*M* EDTA, pH 6.0, and 0.2% DM.
6. HPLC buffer: 20 m*M* Tris-HCl, pH 8.0, 0.2 *M* Na_2SO_4, and 0.15%DM

2.4. Buffers for AE1MD Purification

1. Hemolysis buffer: 0.1 *M* phosphate buffer, pH 7.4, 0.5 m*M* EDTA, 1 m*M* PMSF.
2. Stripping buffer: 15 m*M* NaOH, pH 12, 2 m*M* EDTA, and 0.2 m*M* PMSF.
3. Solubilization buffer: 20 m*M* imidazole, pH 7.0, 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* PMSF, 10% glycerol, and 1% dodecylmaltoside (DDM) or 1% $C_{12}E_8$ detergent.
4. Loading buffer: 20 m*M* imidazole, pH 7.0, 1 m*M* EDTA, 0.5 m*M* PMSF, 1 m*M* NaN_3, 10% glycerol, and 0.1% DDM or $C_{12}E_8$.
5. HPLC buffer: 20 m*M* Tris-HCl, pH 8.0, 0.2 *M* Na_2SO_4, and 0.05% DDM.

3. Methods

3.1. Purification of Glut1 Protein

1. Source: Human erythrocyte "ghost" membrane is prepared from packed outdated red cells (obtained from Bloodbank). Wash cells twice in PBS buffer. Centrifuge at 3000*g* (4000 rpm in GS-3 rotor) for 30 min and collect pellet.
2. Ghost preparation: Lyse cells directly in ice-cold haemolysis buffer. Wash cells continuously in hemolysis buffer and centrifuge at 9000*g* for 15 min at 4°C. Repeat the washing step 10 times until the membrane appears white.

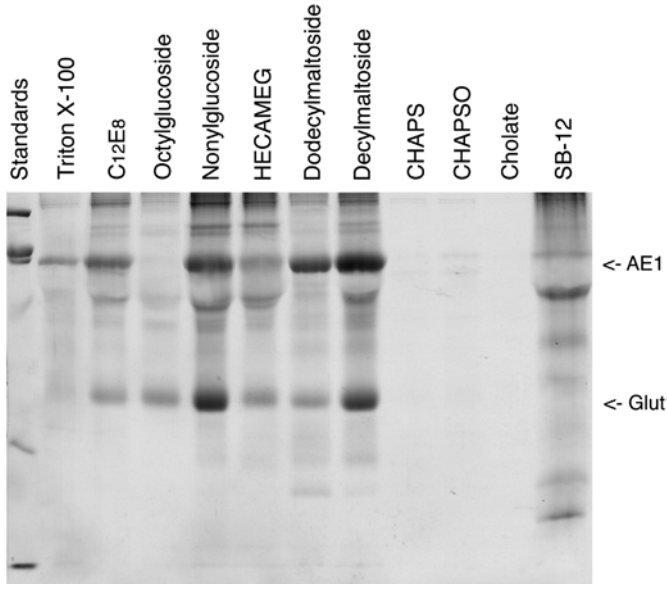

Fig. 1. Selection of detergent for solubilization of human erythrocyte membrane and extraction of Glut1 and AE1MD. The Coomassie Blue-stained SDS-PAGE shows the erythrocyte membrane solubilized with different detergents. PNGase F is included in the SDS buffer to remove the oligosaccharide and sharpen the protein bands. Of all the detergents tested here, DM and NG are most effective at extracting Glut1. NG, however, causes aggregation of the glucose transporter protein and DM is, therefore, selected for solubilization and subsequent purification of the protein. DDM and $C_{12}E_8$ are selected for extraction and purification of AE1MD.

3. Cell stripping: Remove cytoskeletal and peripheral membrane proteins from ghost membrane by washing with 10 vol of stripping buffer for 30 min at 37°C. Centrifuge at 16,000*g* for 20 min. Store aliquoted membrane at −20°C. Typically, one unit of packed red blood cells produces 20 mL of stripped ghost membrane.
4. Erythrocyte membrane solubilization (*see* **Notes 1** and **2**): Solubilize 4 mL of stripped ghost membrane in 20 mL solubilization buffer. Stir solution for 30 min at 4°C. Remove unsolubilized materials and large aggregates by centrifuging at 10,000*g* for 15 min (*See* **Fig. 1**).
5. Q anion exchange chromatography (*see* **Notes 3** and **4**): Load solubilized sample onto a 1-mL HiTrap Q anion exchange column on FPLC, preequilibrated with loading buffer, at a rate of 0.5 mL/min. Wash with 15-mL loading buffer. Elute sample in the same buffer with a 15-mL linear 0–500 m*M* NaCl gradient at 0.25 mL/min (*see* **Fig. 2**). Collect 0.5 mL fractions.

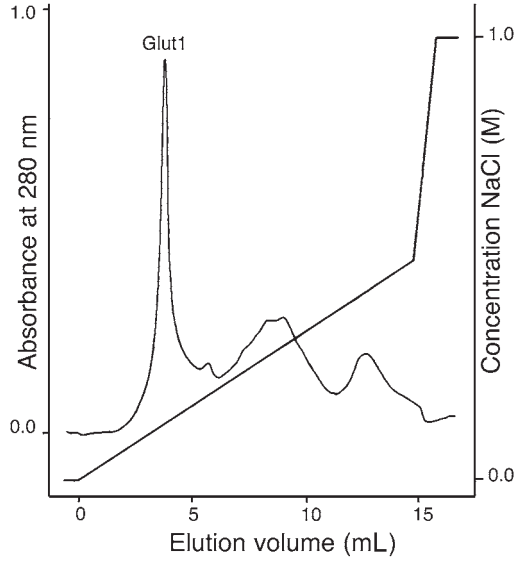

Fig. 2. Elution of DM-solubilized erythrocyte membrane proteins from HiTrap Q anion exchange column. Protein is monitored at 280-nm absorbance and Glut1 eluted at around 100 m*M* NaCl at a concentration of 0.5–1.0 mg/mL. Column buffer is 10 m*M* Bis-Tris pH 6.0, 0.5 m*M* EDTA, 0.2% DM. (Adapted from **ref. *7*** with permission.)

6. Sodium dodecyl Sulfate-polyacxylamide gel electrophoresis (SDS-PAGE) analysis (*see* **Note 5**): To better assess protein purity, deglycosylate Glut1 with 5000 U/mg PNGase F in the presence of 1% SDS at 20°C. Analyze immediately by Coomassie Blue-stained SDS-PAGE (*see* **Fig. 3**).
7. Protein concentration measurement. Determine protein concentration by the Micro BCA assay using spectrophotometer. Include 10 m*M* Bis-Tris in the blank to minimize interference with the assay.

3.2. Purification of AE1 Membrane Domain

1. Source (*see* **Note 6**): The same as **Subheading 3.1.**, **step 1**, or freshly donated human blood.
2. DIDS labeling (*see* **Note 7**): Prepare diluted cells of 25% hematocrit in PBS containing 0.0005% DIDS. Incubate for 60 min at 37°C. Wash cells with PBS and centrifuge to remove unbound DIDS. Cross-link DIDS by incubating with 10 vol of 0.1 *M* $NaHCO_3$, pH 9.5, for 60 min 37°C. Remove excess DIDS from supernatant by centrifuging for 20 min in GS-3 rotor at 7500*g*).
3. Ghost preparation: Lyse DIDS-labeled or unlabeled cells as described in **Subheading 3.1., step 2**.

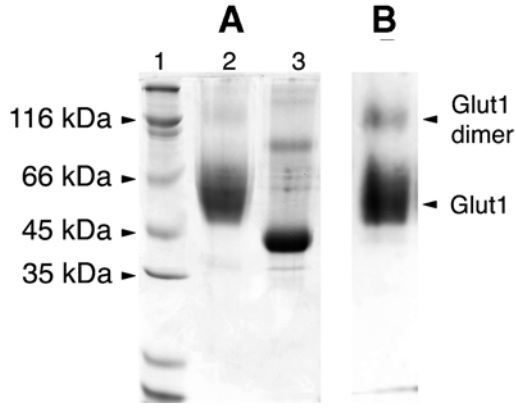

Fig. 3. Coomassie Blue-stained SDS-PAGE showing Glut1 purification and deglycosylation. **(A)** Lane 1: Protein molecular weight standards. Lane 2: Glut 1 purified in DM detergent by HiTrap Q anion-exchange column, with a purity of approx 95% purity. The protein is highly glycosylated. Lane 3: After PNGase F treatment in the presence of SDS, polysaccharide is cleaved from Glut1, resulting a sharpened band that shifted to 43 kDa. **(B)** When Glut1 is incubated with PNGase F, followed by separation from PNGase F using a size-exclusion column, and then loaded onto SDS-PAGE, the broad band of glycosylated Glut1 monomer remains intact. Minimal amounts of Glut1 dimer can form owing to delipidation. 10 μg protein is loaded in each lane. (Reproduced from **ref. *7*** with permission.)

4. AE1 membrane domain generation: Dilute ghost membrane two-fold with hemolysis buffer lacking PMSF. Add trypsin to a final concentration of 5 μ/mL. Stir for 60 min at 4°C, and then add PMSF to a final concentration of 2 m*M* to stop the trypsin digestion. Centrifuge for 15 min 35000*g*. Wash with 10 vol. of hemolysis buffer. Centrifuge again and collect pellet.
5. Cell stripping (*see* **Note 8**): Repeat twice the stripping procedure as described in **Subheading 3.1., step 3** but at 4°C. Store membrane at −20°C.
6. Erythrocyte membrane solubilization (*see* **Notes 1** and **2**): Solubilize 4 mL ghost membrane in 20 mL of solubilization buffer. Stir for 30 min at 4°C. Centrifuge to remove unsolubilized materials (35,000 rpm in Ti45 rotor for 30 min).
7. First anion exchange column (*see* **Note 9**): Load solubilized membrane onto a 5-mL HiTrap Q anion chromatography column on FPLC, preequilibrated with loading buffer, at a rate of 0.5 mL/min. Wash with 5 mL loading buffer. Elute protein with the same buffer, using a 20-mL linear 0.05–1 *M* NaCl gradient at 0.25 mL/min. Collect 0.5 mL fractions (*see* **Fig. 4**).
8. Protein deglycosylation: Deglycosylate AE1MD with 5 U/mg PNGase F overnight at 20°C. Verify completeness of deglycosylation by SDS-PAGE analysis (*see* **Fig. 4**).

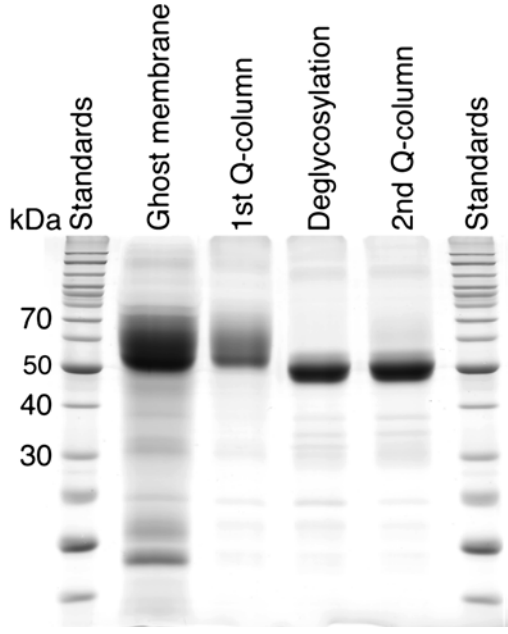

Fig. 4. Coomassie Blue-stained SDS-PAGE showing purification and deglycosylation of human erythrocyte anion exchanger membrane domain. Following purification with the first Q anion exchange chromatography column, the protein is treated with PNGase F to deglycosylated AE1MD and a second Q column is introduced to further purify and delipidate the protein. (Reproduced from **ref. *9*** with permission.)

9. Second anion exchange column: Dialyze deglycosylated peak fractions from the first Q column against column buffer containing 50 m*M* NaCl. Load protein onto a 1-mL HiTrap Q anion exchange chromatography column on FPLC, in the same loading buffer that is used for the first column. The detergent used for the second Q column is DDM (0.1%), undecylmaltoside (UDM, 0.1%), DM (0.1%), decylthiomaltoside (DTM, 0.1%), Cymal-6 (0.25%), Cymal-5 (0.5%), $C_{12}E_8$ (0.1%), $C_{10}E_6$ (0.1%), or C_8E_5 (0.7%). Elute protein from column in five column volumes of loading buffer with a 0.05–2-*M* NaCl linear gradient to ensure high concentration fractions. Collect 0.5 mL fractions (*see* **Fig. 4**).
10. SDS-PAGE analysis (*see* **Note 10**): Analyze protein samples from different purification stages by Coomassie Blue- or silver-stained SDS-PAGE for protein purity.
11. Protein concentration measurements (*see* **Note 11**): Determine protein concentration by the Micro BCA assay using spectrophotometer.

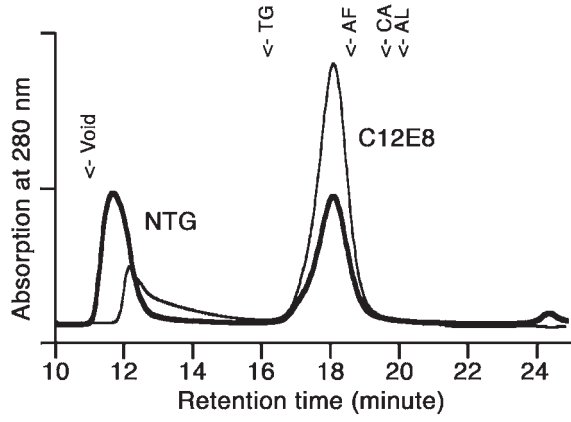

Fig. 5. Size exclusion HPLC chromatograms of AE1MD in $C_{12}E_8$ (thin line) and NTG (thick line) solution. The samples are run on a Shodex KW-804 column, equilibrated with 50 m*M* Tris, pH 8.0, 200 m*M* N_2SO_4, 3 m*M* NaN_3, and 0.1% $C_{12}E_8$. Peaks are identified by their retention time. AE1MD in complex with detergent had a Stokes radius of 66 Å, corresponding to a protein dimer. Elution times of standard proteins of known Stokes radius are indicated: TG = thyroglobulin (86 Å); AF = apoferritin (63 Å); CA = catalase (52Å); AL = aldolase (46Å). (Reproduced from **ref. *9*** with permission.)

3.3. Determination of Stokes Radius of Glut1 and AE1MD by Size-Exclusion HPLC

1. Inject 50 μg of purified Glut1 or AE1MD sample onto a Shodex KW803 or KW804 analytical size-exclusion column on HPLC, preequilibrated with HPLC buffer. Develop in the same buffer at 0.5 mL/min. Monitor UV absorption spectrum at 280 nm (*see* **Note 12**).
2. Record retention times, *t*, for Glut1 and AE1MD (*see* **Fig. 5**).
3. Record retention times of a set of soluble proteins of known Stokes radius (R_S) ***(10)***: thyroglobulin (86 Å), apoferritin (63 Å), catalase (52 Å), and aldolase (46 Å).
4. Determined Stokes radii of Glut1 and AE1MD by comparison with the retention times of standard proteins.

3.4. Determination of Glut1 and AE1MD Stability and Monodispersity

Stability and monodispersity of Glut1 and AE1MD proteins in various detergent solutions and under a wide range of conditions are measured by a modified protocol from the literature ***(11,12)***, using analytical size-exclusion chromatography on HPLC ***(7,9)***. The following parameters can be screened for their effect on protein stability and monodispersity: detergent, pH, additive and temperature. On a Shodex KW803 or 804 column, each such run takes 30 min and only a 50-μg protein sample is needed. Accordingly, a number of conditions can be

screened within a short period of time. Those detergents and conditions that keep the protein stable and monodisperse can be used for its purification and crystallization (*see* **Note 13**).

3.4.1. Sample Preparations

1. To study detergent effect: Incubate 50 μL Glut1 or AE1MD of 1 mg/mL, purified in 0.2% DM or 0.1% $C_{12}E_8$, respectively, in the presence of various detergents at concentrations 0.2% above their respective CMC (added from 10% stock solutions) for 2 to 16 h at 25°C.
2. To study pH effect: Titrate 50 μL samples of purified Glut1 (in 10 m*M* Bis-Tris, pH 6.0), or AE1MD (in 20 m*M* imidazole, pH 7.0), to the desired pH by adding 5 μL of 1 *M* buffer relevant pH. Incubate for 2 to 16 h at 25°C. Use following buffers: pH 4: acetate/acetic acid, pH 5: acetate/acetic acid, pH 6 (control): Bis-Tris-HCl, pH 7: Bis-Tris-HCl, pH 8: Tris-HCl, pH 9: Tris-HCl, pH 10: carbonate-HCl.
3. To study additive effect: Add following additives to purified Glut1 samples: glycerol to 20% (v/v), cytochalasin B to 10 m*M* (from a 2-m*M* stock in ethanol, stored at –20°C), glucose to 100 m*M*, adenosine triphosphate (ATP) to 1 m*M*, and dithiothreitol (DTT) to 5 m*M*. Incubate for 16 h at 37°C.
4. To study lipid effect: Delipidate Glut1 by size-exclusion HPLC and collect protein fractions. Add individual phospholipids to protein samples to a final concentration of 0.1 mg/mL. Incubate for 16 h at 4°C.
5. To study temperature effect: Incubate purified Glut1 and AE1MD samples at 4, 25, and 37°C. Withdraw aliquots at specific time intervals.

3.4.2. Protein Monodispersity Analysis by Size-Exclusion HPLC

1. Load 50 μg of sample, prepared as described above, onto a Shodex KW803 or KW804 size-exclusion column equilibrated with HPLC buffer. Develop at 0.5 mL/min.
2. Collect absorption spectrum at 280 nm. Monitor shape and height of protein peak.
3. Compare with control sample (*see* **Fig. 5**). Extract and integrate chromatograms and calculate proportion of the protein in the peak relative to the total amount (*see* **Tables 1–3**) (*see* **Notes 14–17**).

3.5. Protein Homogeneity Analysis by Electron Microscopy (EM)

Transmission electron microscopy (EM) of negatively stained specimens is a convenient way of assessing the homogeneity of a membrane protein preparation ***(13)***, provided an EM facility is accessible.

1. Specimen preparation: Apply a 2-μL aliquot of purified protein sample (concentration of about 1 mg/mL) to a carbon-coated electron microscopy grid. Remove sample from grid with filter paper after 10. Stain grid twice with 2% uranyl acetate, for 20 s each time. Dry grid thoroughly with filter paper.

Table 1
Monodispersity of Purified Glut1 Protein Under Various Conditions

Reagent	Conditions	% monomer[a] remaining	Effect on monodispersity
(A) Detergents:			
DM	25°C	22	control
NM	overnight	22	–
UDM	without	22	+/–
DDM	glycerol	25	+
DTM		26	+
Cymal-3		19	–
OG		5	–
NG		4	–
DG		13	–
$C_{12}E_8$		6	–
$C_{10}E_8$		4	–
MEGA–10		7	–
(B) pH:			
4.0	25°C	10	–
5.0	overnight	70	++
6.0		95	+++
7.0		90	+++
8.0		65	++
9.0		15	–
(C) Additives:			
None	37°C	24	control
20% Glycerol	overnight	59	++++
10μM Cytochalasin B		42	+++
100mM Glucose		36	++
1mM ATP		19	–
5mM DTT		24	–/+
(D) Lipids:			
None	4°C	61	control
Phosphatidylcholine	overnight	75	+
Phosphatidylethanolamine		56	–
Sphingomyelin		63	+/–
Cholesterol		59	–
Phosphatidylinositol		79	++
Phosphatidylserine		93	+++
Total Erythrocyte Lipids		81	++

[a]Effect of (A) detergent, (B) pH, (C) additives, and (D) lipids on the stability of Glut1. Concentrations of reagents areas follows: 0.1 mg/mL lipid (added to delipidated Glut1), 1% detergent. The proportion of monomeric Glut1 is 100% before treatment. Each parameter is assayed separately, and combination of the positive factors identified in such a way is used to preserve Glut1 monodispersity for an extensive period of time. (Reproduced from **ref. 7** with permission from Academic.)

Table 2
Effects of Detergents on Monodispersity of AE1 Membrane Domain

		Protein without DIDS		Protein with DIDS bound	
Detergent	CMC (%)	% detergent	% dimer remaining[b]	% detergent	% dimer remaining
$C_{12}E_8$[a]	0.0038	0.2	33	0.2	77
$C_{10}E_6$	0.038	0.1	0	0.2	50
C_8E_5	0.21	–	–	1.5	0
DDM	0.008	0.2	17	0.2	79
UDM	0.029	0.2	24	0.2	77
DM	0.8	0.2	6	0.2	71
DTM	0.045	0.2	12	0.2	70
Cymal-6	0.028	–	–	0.1	78
Cymal-5	0.118	–	–	0.3	76
Cymal-4	0.36	–	–	0.7	50
Cymal-3	1.6	2.6	7	–	–
DTG	0.032	–	–	0.1	73
NTG	0.093	–	–	0.1	50
MEGA-9	0.83	–	5	2.0	17
DHPC	0.067	–	–	0.2	73
MO	–	–	–	0.1	74
MP	–	–	–	0.1	76

[a]C_8E_5, pentyloxyethylene octylcylether; $C_{10}E_6$, hexyloxyethylene decylether; $C_{12}E_8$, octyloxyethylene dodecylether; Cymal-3, cyclohexyl–propylmaltoside;-propylmaltoside; Cymal-4, cyclohexyl-butylmaltoside; Cymal-5, Cyclohexyl-pentylmaltoside; Cymal-6, cyclohexyl-hexylmaltoside; DDM, dodecylmaltoside; DHPC, diheptanoyl phosphatidylcholine; DM, decylmaltoside; DTM, decylthiomaltoside; MO, monooleoyl glycerol; MP, monopalmitoyl glycerol; UDM, undecylmaltoside.

[b]Purified AE1MD in 0.1% $C_{12}E_8$ is incubated at for 60 min 37°C in the presence of a second detergent at a concentration 0.1–0.2 % above its critical-micellar-concentration and analyzed by size-exclusion chromatography on HPLC. (Reproduced from **ref. *9*** with permission from Academic.)

Table 3
Effects of pH on Monodispersity of AE1 Membrane Domain with DIDS Bound

pH	Buffer[a]	% dimer remaining[b]
4.5	Acetate	3
5.5	Citrate	72
6.0	Bis-Tris	72
7.0	Imidazole	80
7.0	Phosphate	80
7.5	HEPES	80
7.5	Tris-HCl	82
8.5	TAPS	82
9.0	Glycine	81
10.0	Glycine	81

[a]Buffers are prepared at a concentration of 1.0 *M*.

[b]50 μg samples of purified AE1MD (10 m*M* imidazole, pH 7.0) are titrated to the relevant pH by addition of 5 μL of 1 *M* buffer of the desired pH, and then incubated at 25°C for 30 min, followed by analysis using size-exclusion chromatography on HPLC. (Reproduced from **ref. *9*** with permission from Academic.)

2. Electron microscopy: Mount grid onto a room-temperature, single-tilt holder. Insert into electron microscope. Search at low magnification (2000–3000). Observe and record electron micrographs at higher magnification (40,000–100,000) (*see* **Fig. 6**).

4. Notes

1. Detergent selection: A variety of detergents are tested for extraction of Glut1 from erythrocyte membrane (*see* **Fig. 1**). They include Triton X-100, $C_{12}E_8$, octylglucoside (OG), nonylglucoside (NG), HECAMEG, DDM, DM, CHAPS, CHAPSO, sodium cholate and SB-12. For this purpose, 50 μL membrane in 50 m*M* Tris/HCl pH 7.4, 0.5 m*M* EDTA, is solubilized with 1% micellar detergent, i.e., at a detergent concentration 1% above its critical micellar concentration (CMC). After incubation at 4°C for 30 min with stirring and removal of unsolubilized membrane, 25 μL of the supernatant is taken for SDS-PAGE analysis, followed by Coomassie blue staining. Of all the detergents tested here, DM and NG are most effective at extracting Glut1. However, NG causes aggregation of the glucose transporter protein, and DM is, therefore, selected for solubilization of Glut1 and subsequent purification. For comparison, four detergents extract AE1 well: $C_{12}E_8$, NG, DDM, and DM. Among them, the two with long carbon-chain (C12), $C_{12}E_8$ and DDM, keep the protein stable and are therefore used for AE1MD extraction and purification.
2. Membrane solubilization completeness: It is critical that the ghost membrane is completely solubilized for subsequent protein purification—the solution should become translucent following 30 min stirring at 4°C in the presence of 1–2%

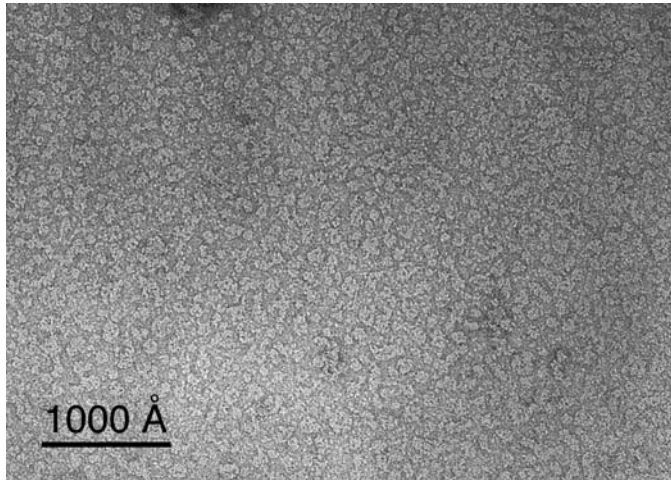

Fig. 6. EM of purified Glut1 sample. Purified Glut in DM detergent is very homogeneous. The protein sample is negatively stained with 2% uranyl acetate on carbon-coated EM grid. The protein molecules adhere to the carbon support film in various orientations, resulting in different shapes and sizes. Collapsing of the protein molecule on the grid and the surrounding detergent micelle make the protein's dimensions appear to be somewhat larger than in solution, scale bar represents 1000 Å.

micellar detergent. Otherwise add 20% more solubilization buffer and stir the solution for another 15 min.

3. Glut1 purification yield: For Glut1 purification, erythrocyte membrane is completely solubilized by 2% DM. In this detergent, Glut1 binds to the HiTrap Q anion exchange column at pH 6.0, and is eluted as a sharp peak at approximately 100 m*M* NaCl, with a protein concentration of 0.5–1.0 mg/mL (*see* **Fig. 2**). Other erythrocyte membrane proteins, such as AE1, do not bind significantly to the column under these conditions and, therefore, the Glut1 peak dominates the chromatogram, yielding a positive purification procedure. The protein in the peak fractions is approx 95% pure. The yield of the Glut1 peak is 5 ± 1% (n=5) of total protein in the stripped erythrocyte membrane, although the entire yield of Glut1, including non-peak fractions, is estimated to be 8–10% of total protein.
4. Glut1 binding to Q column: The binding of Glut1 to an anion exchange matrix at pH 6.0 needs explanation, given the high isoelectric point of Glut1 of around 8.4 ***(14)***. At this pH, Glut1 should be positively charged (cationic) and should not bind to an anion exchange column. We hypothesize that negatively charged lipids copurifying, such as phosphatidylserine and phosphatidic acid, could bridge the positively charged protein and the positively charged column matrix, resulting in the binding of Glut1 to the anion exchange resin. Interestingly, the Q column-purified Glut1 will not bind to the same anion exchange column again, but will bind weakly to a cation exchange column instead, suggesting that the localized negative charges

holding the protein to the first Q column have become dispersed upon release from the resin.

5. Glut1 deglycosylation: PNGase F is added to purified Glut1 sample at 5000 μ/mg protein and subsequently loaded onto SDS-PAGE. Consequently, the Glut1 band becomes sharpened and shifts to about 43 kDa, indicating complete deglycosylation of the protein (*see* **Fig. 3A**). However, the amount of glycosidase needed is 1000 times more than that needed to deglycosylate AE1MD. When Glut1 is separated from PNGase F using a size-exclusion column following incubation, the broad band of glycosylated Glut1 on SDS-PAGE remains intact (*see* **Fig. 3B**). We, therefore, conclude that PNGase F is only able to deglycosylate Glut1 when the protein is unfolded in the presence of SDS and is inactive against folded Glut1.
6. AE1MD purification: The purification protocol presented here differs from the protocol of Casey et al. ***(8)*** in several ways. An additional membrane-stripping step is employed here to further remove contaminating proteins. A strong anion exchanger resin, instead of diethylaminoethyl (DEAE), is used in the first chromatography step and the pH in the column is changed from 8.0 to 7.0, resulting in less background contaminant. Furthermore, the newly introduced second Q column removes detached oligosaccharide and the glycosidase used, and further delipidates the protein.
7. AE1MD DIDS-labeling: The covalently bound DIDS locks AE1 into a fixed conformation and stabilizes the protein. Mild trypsinization cleaves off the cytosolic 42 kDa domain from the membrane domain. Both are necessary for three-dimensional (3-1) crystal formation of the protein ***(9)***.
8. Membrane stripping: Repeating the membrane stripping step twice removes a large proportion of glycophorin A, a single-span membrane protein that otherwise contaminates the preparation. This is particularly important because its presence is difficult to detect by Coomassie Blue-stained SDS-PAGE.
9. AE1MD purification and deglycosylation: Both $C_{12}E_8$ and DDM completely solubilize the stripped red cell membrane and effectively extract AE1MD from the membrane. The first HiTrap Q anion exchange column produced 90% pure AE1 membrane domain (*see* **Fig. 4**). Deglycosylation of AE1MD with PNGase F at 5 U/mg protein overnight at 20°C cleaves off the oligosaccharide from the protein completely. Unlike in the case of Glut1, the deglycosylation reaction does occur on the intact AE1MD in the absence of SDS, yielding a markedly more homogeneous preparation. AE1MD elutes from the second Q column as a sharp peak at 0.8 *M* NaCl, and the steep salt gradient results in concentrated (1–2 mg/mL) AE1MD fractions at a relatively low (0.1%) detergent concentration. The final protein purity is close to 95%. In addition, this Q column can also be used to exchange detergent for subsequent crystallization experiments.
10. Membrane protein SDS-PAGE analysis: In contrast to soluble proteins, membrane protein samples prepared for SDS-PAGE should not be boiled. Boiling the samples in SDS causes severe aggregation. Prolonged incubation of the samples with the sample buffer at the room temperature, or even 4°C, also leads to aggregation. The best results are obtained with freshly prepared samples on newly cast gels. Ready-made gels from commercial sources often yield blurred bands. In addition, hydrophobic proteins with multiple transmembrane segments like Glut1 and

AE1MD tend to migrate to lower positions on SDS-PAGE, compared with soluble proteins with similar molecular weights. This is probably due to the larger amounts of SDS bound to the polypeptide or partially folded structures of the membrane proteins in SDS.

11. Membrane protein concentration measurement: We have tested various protein assays for protein concentration determination, including Lowry, Bradford, BCA, and Micro-BCA. Micro-BCA from Pierce has the broadest compatibility with detergents, and it produced very consistent results, within 15% from those measured by absorption at 280 nm. We therefore choose this assay for protein concentration determination.
12. Stokes radius measurement: From a size-exclusion chromatography column on HPLC, both Glut1 in DM and AE1MD in DDM elute as a single major protein peak (*see* **Fig. 5**). By comparison with the retention times of soluble protein standards, the Stokes radii of the Glut1 and AE1MD particles are determined to be 50 Å and 66 Å, respectively. Such Stokes radii are of the protein-detergent complexes, not of the proteins along. Compared with the Stokes radii of other membrane transporters with similar molecular weights ***(11,15)***, the Glut1 protein is likely to be a monomer in DM and AE1MD a dimer in DDM.
13. Protein monodispersity and stability: To maintain the proteins in a monodisperse state for crystallization, various factors are screened for their ability to influence the stability of the protein. They include detergent, pH, additive, lipid, and temperature. Conditions are eventually found to maintain the protein monodisperse for weeks (*see* **Tables 1–3**).
14. Detergent effect: Detergents can be assayed for their effect on the stability of Glut1 and AE1MD in solution. Maltoside detergents generally retain Glut1 monodispersity, with DTM being the best, followed by DM and DDM (*see* **Table 1A**). Similarly, AE1MD in complex with DIDS is also particularly stable in maltoside detergents, including: DDM, UDM, DM, DTM, Cymal-6, Cymal-5, and Cymal-4 (*see* **Table 2**). In addition, it remains monodisperse in long-chain C_mE_n and glucoside detergents like $C_{10}E_6$, decylthioglucoside (DTG), and nonylthioglucoside (NTG). Short-chain detergents, including C_8E_5, octylthioglucoside (OTG) and heptathioglucoside (HTG), however, destabilize AE1MD.
15. pH effect: A pH range of 6–7 is optimal for stabilizing the Glut1 protein at 25°C (*see* **Table 1B**). In contrast, the DIDS-bound dimeric AE1 membrane domain stays monodisperse over a wider pH range, from 5.5 to 10.0 (*see* **Table 3**),
16. Additive effect: Several reagents can prevent the Glut1 and AE1MD from aggregating and thus increased their effective lifetime (*see* **Table 1C** and **D**). Among them, glycerol at 20–30% is most effective, increasing the stability of both proteins significantly. This is in agreement glycerol's stabilizing effect for soluble proteins ***(16)***. We, therefore, include glycerol in all solubilization and purification steps. The inhibitor, cytochalasin B, also has a positive effect for Glut1, as does glucose.
17. Temperature effect: Combination of the stabilizing factor identified above results in conditions that maintain Glut1 and AE1MD monodispersity over a wide temperature range. At 4°C Glut1 undergoes minimal amounts of aggregation over time. Moreover, in the presence of 20% glycerol, Glut1 could be stored for at

least 6 mo at –20°C without any apparent aggregation. Freezing, however, causes irreversible aggregation of AE1MD. Assuming a monoexponential decay, the half-life of the monodisperse Glut1 in DM at pH 6 (with 50% monomer retained) is calculated to be 13 h, 8 d, and 5 wk, respectively, at 37, 25, and 4°C.

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Purification of the Human Erythrocyte PS-Stimulated Mg^{2+}-ATPase

A Putative PS Flippase

Jill V. Lyles, Kathleen Cornely-Moss, Christine M. Smith, and David L. Daleke

1. Introduction

The transbilayer distribution of phospholipids across the erythrocyte membrane is asymmetric; the amine-containing phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are enriched on the cytoplasmic surface of the membrane, whereas the choline-containing lipids, phosphatidylcholine and sphingomyelin, are enriched on the external surface ***(1–4)***. In contrast to other phospholipids, the distribution of PS is absolute and is restricted entirely to the inner monolayer. This arrangement of lipids is generated by asymmetric biosynthesis and is maintained by a combination of passive (slow spontaneous flip-flop) and active transport processes. The distribution of phosphatidylserine is preserved by vanadate-sensitive, adenosine triphosphate (ATP)-dependent transport from the outer-to-inner monolayer. The protein responsible for this activity has been coined the aminophospholipid "flippase." It is highly selective for phosphatidylserine and displays a strict requirement for the structure of its preferred substrate. Although the flippase transports *N*-methyl-PS, additional modification or deletion of the amine or carboxyl groups significantly reduces substrate transport ***(5,6)***. Flippase activity is absolutely dependent on the stereoconfiguration of the *sn*-2 position of the glycerol backbone, but not of the serine amino acid headgroup ***(5–8)***. In addition to these substrate requirements, flippase activity is also sensitive to sulfhydryl reagents and Ca^{2+} ***(9–11)***. These properties have provided the biochemical basis for the identification and purification of a potential erythrocyte flippase.

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The isolation of this enzyme from human erythrocytes has been reported independently by several laboratories ***(5,12–19)***. Each of these preparations differ somewhat in physical properties, yet all are stimulated by PS (for a recent review, *see* **ref. *20***). What follows is a detailed description of the protocol used by our laboratory. It is based on methods previously developed for the purification of ATPases with similar biochemical characteristics from bovine brain ***(16)*** and bovine chromaffin granules ***(15,21)*** and has been significantly modified by our laboratory and others. Erythrocyte membranes are isolated from units of whole blood or packed red blood cells, solubilized with detergent and purified by sequential ion exchange (Q Sepharose, Mono Q) chromatography. Vanadate-sensitive PS-stimulated ATPase activity is used to identify putative flippase-containing fractions.

2. Materials

In addition to typical biochemical instrumentation, an automated preparative-scale protein chromatography system [fast protein liquid chromatography (FPLC) or equivalent] is required. The preparation of buffers and solutions requires approx 80 L of deionized, distilled H_2O. All buffers should be filtered (0.45 μm, type HA, Millipore Corp., Bedford, MA), degassed, and equilibrated to 4°C.

2.1. Preparation of Human Erythrocyte Membranes

1. Two units of fresh (<1 wk) whole blood or human erythrocytes, stored at 4°C (*see* **Note 1**).
2. Isotonic saline: 0.15 *N* NaCl (4 L) (*see* **Note 2**).
3. Lysing buffer (buffer A): 10 m*M* Tris-HCl, 1 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0 (10 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
4. Erythrocyte membrane washing buffer (buffer A without EDTA): 10 m*M* Tris-HCl, pH 8.0 (4 L).
5. Tangential flow filtration device (Pellicon) fitted with an HVMP 0.45 μm, 5 ft^2 cassette (Millipore Corp., Bedford, MA).
6. Tangential flow device and membrane cleaning solution: 0.1 *N* NaOH (2 L).
7. Tangential flow device and membrane storage solution: 0.05% NaN_3 in H_2O (4 L).

2.2. Erythrocyte Membrane Protein Extraction

1. Membrane solubilization buffer (5X): 50 m*M* Tris-HCl, 0.5 m*M* $CaCl_2$, 5% (v/v) $C_{12}E_9$ (0.1 L) (*see* **Note 4**). Add protease inhibitors (*see* **Note 3**) immediately prior to use.

2.3. Calmodulin-Agarose Chromatography

1. Calmodulin agarose (20 mL, Sigma Corp., St. Louis, MO) and an appropriately sized column.

2. Calmodulin agarose equilibration buffer: 20 m*M* Tris HCl, 75 m*M* KCl, 1 m*M* $MgCl_2$, 100 μ*M* $CaCl_2$, 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.0 (0.5 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
3. Calmodulin agarose elution buffer: 20 m*M* Tris HCl, 75 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* EDTA, 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.0 (0.5 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
4. Storage buffer: calmodulin agarose elution buffer + 0.05% NaN_3.

2.4. Q Sepharose Chromatography

1. Q Sepharose fast flow anion exchange media (500 mL, Amersham Biosciences Corp., Piscataway, NJ) and an appropriately sized column.
2. Buffer B: 10 m*M* Tris-HCl, 0.1 m*M* ethylene glycol-bis (2-aminoethylether)-N.N.N.'N'-tetraacetic acid (EGTA), 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.4 (12 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
3. Buffer B + 1 *M* NaCl: 10 m*M* Tris-HCl, 0.1 m*M* EGTA, 1 *M* NaCl, 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.4 (4 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
4. Q Sepharose storage buffer: 10 m*M* Tris-HCl, 0.1 m*M* EGTA, 0.5 *M* NaCl, 0.1% $C_{12}E_9$, 10% glycerol, 20% ethanol, pH 7.4 (4 L).

2.5. Mono Q Chromatography

1. Mono Q 5/5 anion exchange column (Amersham Biosciences Corp., Piscataway, NJ).
2. Buffer B: 10 m*M* Tris-HCl, 0.1 m*M* EGTA, 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.4 (0.1 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
3. Buffer B + 1 *M* NaCl: 10 m*M* Tris-HCl, 0.1 m*M* EGTA, 1 *M* NaCl, 0.1% (v/v) $C_{12}E_9$, 10% (v/v) glycerol, pH 7.4 (0.1 L). Add protease inhibitors (*see* **Note 3**) immediately prior to use.
4. Mono Q storage buffer: 10 m*M* NaOH (10 mL).

2.6. Protein Assay (Modification of the BCA Assay [23])

1. Reagent A: 0.16% (w/v) sodium potassium tartrate, 1.6% (w/v) NaOH, 8% (w/v) Na_2CO_3 in H_2O, pH 11.25. Store at room temperature.
2. Reagent B: 4% (w/v) 4,4'-dicarboxy-2,2'-biquinoline, disodium salt (BCA). Store at room temperature.
3. Reagent C: 4% (w/v) cupric sulfate pentahydrate in H_2O. Store at room temperature.
4. Trichloroacetic acid: 12% (w/v) in H_2O. Store at 4°C.
5. Deoxycholate: 1% (w/v) in H_2O. Store at 4°C
6. Protein standard: 1 mg/mL bovine serum albumin (BSA) in H_2O. Store at –20°C

2.7. ATPase Assay (Adapted from Lanzetta et al. [22])

1. ATPase assay buffer stock solutions (store at 4°C):
   a. Buffer cocktail (10X): 625 m*M* HEPES, 125 m*M* $MgCl_2$, 12.5 m*M* EGTA, pH 7.4.
   b. Ouabain: 5 m*M* in H_2O.
   c. $C_{12}E_9$: 5% (v/v) in H_2O.

d. Sodium orthovanadate: 5 m*M* in H_2O. Carefully adjust to pH 10.5 and check concentration by measurement of A_{260} ($\varepsilon = 3.55$ mM^{-1}). Store at 4°C.
2. 1-Palmitoyl-2-oleoyl-sn-3-glycerophosphoserine (POPS, Avanti Polar Lipids, Alabaster, AL) in $CHCl_3$. Store at −20°C.
3. ATP stock (100 m*M* in H_2O): Titrate to pH 7.0 with 10 *N* NaOH and check concentration by measurement of A_{260} ($\varepsilon = 15.3$ mM^{-1}). Store in aliquots at −20°C.
4. Color reagent solutions (store at room temperature):
   a. Ammonium molybdate: 4.5% (w/v) in 4 HCl.
   b. Malachite green hydrochloride: 0.045% (w/v) in H_2O.
   c. Sterox™ flame photometer diluent (Bacharach, Pittsburgh, PA).
   d. Citric acid: 34% (w/v) in H_2O.
5. Phosphate standard: 1 m*M* sodium phosphate in H_2O. Store at room temperature.

2.8. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Acrylamide monomer solution: 29% acrylamide, 1% *bis*-acrylamide in H_2O. Store at 4°C for up to 3 mo.
2. Separating gel buffer: 1.5 *M* Tris-HCl, 0.4% (w/v) SDS pH 8.8. Store at room temperature.
3. Stacking gel buffer: 0.5 *M* Tris-HCl, 0.4% (w/v) SDS, pH 6.8. Store at room temperature.
4. Electrode buffer: 0.025 *M* Tris-HCl, 0.1% SDS, 0.192 *M* glycine, do not adjust pH (should be approx pH 8.3). Store at room temperature.
5. Ammonium persulfate: 10% (w/v) in H_2O. Store at 4°C for up to 1 mo.
6. Sample buffer (4X): 0.25 *M* Tris-HCl, 8% SDS, 0.008% bromophenol blue, 40% glycerol, pH 6.8. Store at −20°C in 0.8-mL aliquots. Before use, thaw and add 0.2 mL β-mercaptoethanol.
7. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED).

3. Methods

The following protocol typically produces a 50–70-fold purification of an 80kDa vanadate-sensitive PS-stimulated ATPase with a specific activity of 300–560 nmoles/min/mg (*see* **Table 1**). Starting with 2 U of whole blood (approx 1 g membrane protein) the yield is typically 500 μg of protein (2.8% yield). Unless otherwise indicated all steps are carried out at 4°C.

3.1. Preparation of Human Erythrocyte Membranes

1. Centrifuge whole blood or packed erythrocytes at 5000*g* for 5 min in 500-mL polycarbonate tubes (Beckman JA-10 rotor). Remove plasma and buffy coat (mostly white blood cells) by aspiration. Wash erythrocytes twice more with 0.15 *N* NaCl. After each wash aspirate and discard the clear supernatant, as well as any remaining buffy coat.
2. Prepare the tangential flow filtration device by pumping 20 L filtered H_2O, followed by 2 L Buffer A, through the system to wash the filter.

Table 1
Purification of the Human Erythrocyte Vanadate-Sensitive PS-Stimulated Mg^{2+}-ATPase[a]

Sample	Protein (mg)	Units (nmol Pi min^{-1})	Specific activity (nmol Pi min^{-1} mg^{-1})	Purification (fold)
Membranes	955.0	5582.3	5.8	1.0
Solubilized membranes	330.5	2481.6	7.5	1.3
CaM agarose eluate	229.6	2088.7	9.1	1.6
Q Sepharose pool	13.5	597.9	44.4	7.6
Mono Q pool	0.5	154.6	297.9	51.0

[a]Data are the average of five independent purifications.

3. To lyse the erythrocytes, fill a 4-L holding tank with 2–3 L Buffer A (containing protease inhibitors) and add the washed erythrocytes. Incubate on ice, with occasional stirring for 30 min.
4. With the tangential filtration device in constant volume mode, wash the erythrocyte membranes with an additional 8 L Buffer A, followed by 6–8 L Buffer A without EDTA (*see* **Note 5**). Once the erythrocyte membranes are white, pump them out of the tangential flow device into 500-mL polycarbonate centrifuge tubes. Centrifuge at 17,700*g* for 50 min (Beckman JA-10 rotor). Aspirate the supernatant and carefully discard the "button" under the membrane pellet. Alternatively, erythrocyte membranes can be washed by centrifugation, rather than tangential flow filtration (*see* **Note 6**).
5. After use, rinse the tangential flow filtration device and filter by pumping 20 L filtered H_2O through the system. Clean the membrane by circulating 0.1 *M* NaOH through the apparatus for 30 min, rinse briefly with filtered H_2O, and finally wash with 0.05% NaN_3. Store the membrane and gaskets in 0.05% NaN_3 at 4°C.

3.2. Erythrocyte Membrane Protein Extraction

1. Solubilize membranes with 5X solubilization buffer (4 parts ghosts mixed with 1 part 5X buffer) and incubate on ice for 30 min, with occasional mixing.
2. Remove insoluble material by centrifugation at 96,500*g* (Beckman SW28 rotor) for 45 min. Reserve the supernatant, and if necessary, re-extract the pellets with an equal volume of Buffer A containing 1% $C_{12}E_9$. After centrifugation, combine the supernatants.

3.3. Calmodulin-Agarose Chromatography (see Note 7)

1. Apply the detergent extract to a calmodulin agarose affinity column (20 mL), preequilibrated with calmodulin agarose equilibration buffer, at a flow rate of 1 mL/min. Collect unbound protein.
2. Remove bound protein with calmodulin agarose elution buffer and reequilibrate with five column volumes of calmodulin equilibration buffer.
3. Store the column in calmodulin elution buffer containing 0.05% NaN_3.

3.4. Q Sepharose Chromatography

1. Apply protein that did not bind to the calmodulin agarose affinity column to a Q Sepharose fast flow anion exchange column (500 mL), preequilibrated with Buffer B, at a flow rate of 5–10 mL/min.
2. Wash unbound protein from column with Buffer B until A_{280} returns to background.
3. Elute bound protein with a five column volume (2.5 L) NaCl gradient (0–0.5 *M*) in Buffer B. Collect 25-mL fractions.
4. Assay fractions for PS-stimulated vanadate-sensitive ATPase activity (*see* **Subheading 3.7.**).
5. Pool active fractions. Vanadate-sensitive PS-stimulated ATPase typically elutes between 0.20–0.27 *M* NaCl (*see* **Fig. 1**).
6. Store column in Buffer B + 20% ethanol.

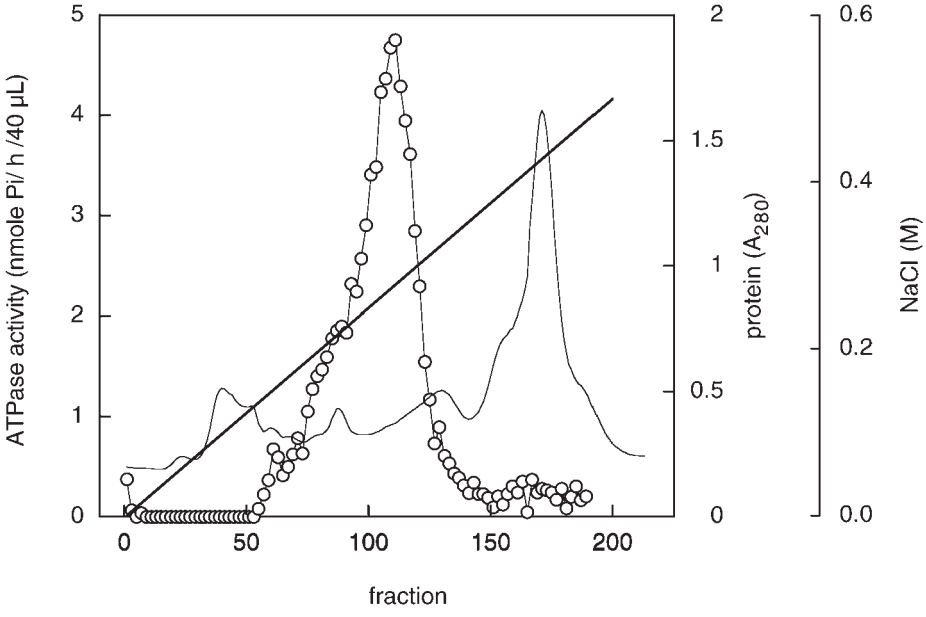

Fig. 1. Q Sepharose anion exchange chromatography. Detergent solubilized human erythrocyte membranes were passed through a calmodulin agarose column and applied to a 500-mL Q Sepharose fast flow column. Unbound protein was removed by washing with Buffer B and bound protein was eluted with a linear NaCl (0–0.5 *M*) gradient (heavy solid line) in Buffer B. Fractions of 25 mL were collected and vanadate-sensitive, PS-stimulated ATPase activity of 40-µL aliquots was measured (open circles). Protein content (light solid line) is expressed as A_{280}.

3.5. Mono Q Chromatography

1. Concentrate the pooled Q Sepharose fractions with an ultrafiltration device, fitted with an Amicon YM-10 membrane (Millipore Corp., Bedford, MA), to a volume of approx 10 mL. Redilute three- to sixfold with Buffer B to lower the NaCl concentration to less than 0.1 *M*.
2. Apply the pooled Q Sepharose fractions to a Mono Q 5/5 column (1 mL), previously equilibrated with Buffer B, at a flow rate of 1 mL/min. Remove unbound protein by washing with Buffer B until A_{280} reaches background. Elute bound protein with a 20-column volume NaCl (0–0.5 m*M*) gradient in Buffer B.
3. Assay fractions for PS-stimulated vanadate-sensitive, PS-stimulated ATPase activity (*see* **Subheading 3.7.**) and protein content (*see* **Subheading 3.6.**).
4. Pool active fractions. Vanadate-sensitive ATPase activity typically elutes at 0.26 *M* NaCl (*see* **Fig. 2**).

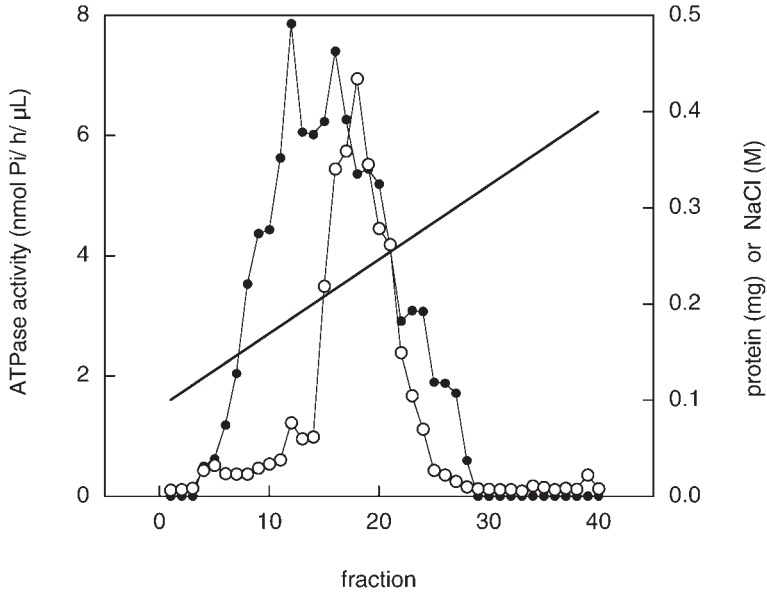

Fig. 2. Mono Q anion exchange chromatography. Pooled Q Sepharose fractions were concentrated by ultrafiltration and applied to a Mono Q 5/5 column. Unbound protein was removed by washing with Buffer B and bound protein was eluted with a linear NaCl (0.1–0.4 *M*) gradient (solid line) in Buffer B. Fractions of 1 mL were collected and vanadate-sensitive, PS-stimulated ATPase activity of 5-µL aliquots was measured (open circles). Protein content (filled circles) of 30-µL aliquots was determined using a modification of the BCA protein assay.

3.6. Protein Assay (Modification of the BCA Assay [23])

1. Prepare bovine serum albumin protein standards (0–15 µg from a 1-mg/mL stock solution), in microcentrifuge tubes. Balance volumes to 15 µL with 0.15 N NaCl.
2. Aliquot the appropriate volume of samples into separate microcentrifuge tubes. Balance volumes to 15 µL with 0.15 *N* NaCl.
3. Add 30 µL of 1% deoxycholate to samples and standards. Incubate at room temperature for 10 min.
4. Add 1 mL 12% ice cold TCA to each tube. Incubate on ice for 30 min.
5. Centrifuge at 16,000*g* in a microcentrifuge for 20 min.
6. Prepare BCA working reagent. Mix 0.2 vol Reagent C and 4.8 vol Reagent B. Add 5 vol Reagent A and 10 vol H_2O. Use the same day.
7. Carefully aspirate supernatants and add 1 mL of BCA working reagent.
8. Incubate samples in a shaking water bath at 60°C for 1 h.
9. Cool to room temperature and measure A_{562} in a spectrophotometer or microplate reader.

3.7. ATPase Assay (Modification of Lanzetta et al. [22])

1. Prepare two assay cocktails each composed of 62.5 m*M* HEPES, 12.5 m*M* $MgCl_2$, 1.25 m*M* EGTA, 0.25 m*M* ouabain, 0.125% $C_{12}E_9$, and 625 μ*M* POPS, but one with and one without 0.25 m*M* vanadate. Aliquot the appropriate volume of POPS in $CHCl_3$ into two 13 × 150 mm test tubes. Remove the solvent with a stream of nitrogen and resuspend the lipid in the required amount of $C_{12}E_9$ plus one-half the required volume of water. Sonicate the cocktail briefly to resuspend the lipid. Add the appropriate amounts of 10× ATPase assay buffer concentrate, 5 m*M* ouabain, 5 m*M* vanadate and the remaining water to yield the concentrations indicated above. Mix well and store on ice.
2. Prepare the ATP working solution. Dilute the 100 m*M* ATP stock solution to 12.5 m*M* with water. Keep on ice.
3. Prepare phosphate standards from a 1 m*M* stock solution in H_2O. Balance standards to 40 μL with H_2O. The linear range of this assay is 0–25 nmol phosphate.
4. Pipet 40 μL of each sample into two separate tubes.
5. Add 40 μL of assay cocktail, one with and without vanadate, to each set of tubes.
6. Incubate on ice for 15 min.
7. Add 20 μL ATP (12.5 m*M*) in timed intervals (20 s) and incubate at 37°C for 1 h.
8. While the samples are incubating, prepare the color reagent working solution. Mix ammonium molybdate:malachite green:Sterox stock solutions in a 1:3:0.08 ratio and let stand for 15 min. Filter through Whatman #2 paper and use within 1 hour.
9. When the incubation is complete, add 800 μL of color reagent in timed intervals, followed 10 s later by 100 μL of 34% (w/v) citrate.
10. Measure A_{660} immediately in timed intervals.
11. Calculate net vanadate-sensitive activity = (ATPase activity−vanadate)–(ATPase activity + vanadate) (*see* **Note 8**).

3.8. SDS-PAGE (24)

1. Prepare 30 mL 8% polyacrylamide separating gel solution by mixing 8-mL acrylamide monomer solution, 7.5-mL separating gel buffer, 14.3 mL H_2O. Add 10 μL TEMED and 150 μL ammonium persulfate. Immediately pour enough solution (approx 27 mL) into a 14 (w) × 16 (h) × 0.15 (d) cm gel frame to obtain a 12-cm-height separating gel and carefully overlay with approx 200 μL H_2O. After the gel has polymerized (approx 1 h), prepare 10 mL 4.5% polyacrylamide stacking gel by mixing 1-5-mL acrylamide monomer solution, 2.5-mL stacking gel buffer, 6 mL H_2O. Add 5 μL TEMED and 50 μL ammonium persulfate. Decant the H_2O from the polymerized separating gel and pour the stacking gel solution into the frame. Fit the frame with a sample well comb and let polymerize (approx 1 h).
2. Mix sample (1–20 μg, depending on choice of protein stain) with the appropriate volume of 4× sample buffer and incubate for at least 10 min at room temperature (*see* **Note 9**).

3. Separate proteins at a constant current of 30 mA.
4. Visualize proteins by Coomassie Blue R-250 or silver ***(25)*** staining (*see* **Fig. 3**).

4. Notes

1. The age of the red cells affects the state of this enzyme. ATPase purified from outdated blood differs in activity and molecular weight from that purified from freshly isolated blood. It is recommended that only fresh whole blood or packed erythrocytes, preferably leukocyte-reduced, be used for starting material.
2. Although each buffer can be made and filtered individually, it is more convenient to prepare buffers from concentrated stock solutions, all equilibrated to 4°C and prefiltered. When adjusting pH at temperatures greater than 4°C, be sure to make appropriate corrections for changes in pKa.
3. Prepare inhibitor stock solutions in advance (working dilutions 1:1000): 1 m*M* leupeptin in water (stable for 1 mo at −20°C); 1 m*M* pepstatin in ethanol (stable for 1 wk at 4°C), 1 M dithiothreitol (DTT) in water (stable for 3 mo at −20°C).
4. The selection of the detergent used for membrane solubilization is critical and was based both on efficiency of membrane extraction and stabilization of enzyme activity. The methods upon which this protocol is founded prescribed $C_{12}E_8$, a nonionic polyoxyethylene detergent. Other transmembrane transporters have been successfully purified using similar detergents, such as Triton X-100. We selected $C_{12}E_9$ owing to its similar physical properties and lower cost. A survey of several detergents revealed that $C_{12}E_9$ was one of the most efficient at solubilizing and stabilizing PS-stimulated ATPase activity (not shown). It should be noted that commercial $C_{12}E_9$ is of varying purity and may contain a mixture of isomers possessing varying alkyl chain lengths. The alkyl chain heterogeneity of the $C_{12}E_9$ used should be assessed.
5. The erythrocyte membranes are washed with Buffer A without EDTA to avoid a reduction in divalent cation content of the solubilization buffer, which would hinder binding of contaminant proteins to the calmodulin column.
6. Erythrocyte membranes can be prepared by centrifugation, rather than tangential flow filtration, by lysing cells in 12 vol of Buffer A on ice for 30 min. Collect the resulting membranes by centrifugation (17,700*g*, 30 min) and wash at least 3× with Buffer A. Successive washings will result in lower yields of membranes. The tangential flow filtration method requires fewer manipulations, is faster, and results in a greater recovery of membranes, but may yield membranes with higher residual hemoglobin content. The final high-speed centrifugation step is necessary to concentrate the membranes maximally prior to detergent solubilization. If more tightly packed membranes are desired, the membranes can be centrifuged for 30 min at 33,000 *g* (Beckman JA-18 rotor).
7. The calmodulin affinity step is not required, but facilitates the removal of the erythrocyte Ca^{2+}-ATPase ***(26)*** and a contaminating protease ***(27)***. In addition to the Ca^{2+}-ATPase, other ATPases can be separated from the PS-stimulated Mg^{2+}-ATPase during the course of this purification, including a vanadate-insensitive ATPase, which elutes from the Q Sepharose column at approx 0.125 *M* NaCl, and

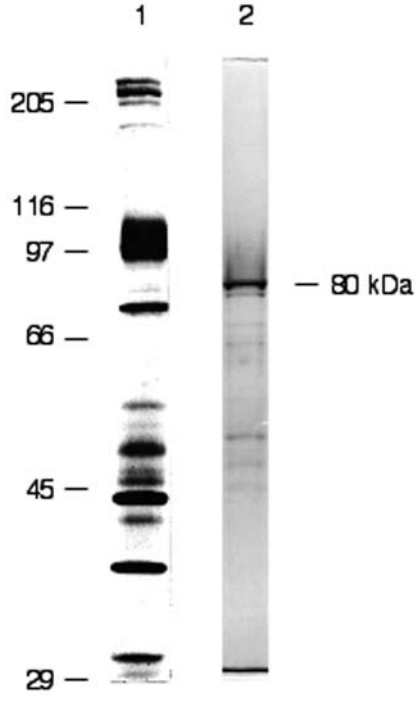

Fig. 3. The human erythrocyte vanadate-sensitive PS-stimulated Mg^{2+}-ATPase. Proteins were separated with an 8% SDS polyacrylamide gel and visualized by silver staining: total erythrocyte membrane proteins (lane 1); purified Mg^{2+}-ATPase (lane 2).

ATP-citrate lyase (120 kDa), which elutes from the Mono Q column at lower salt concentration than the PS-stimulated Mg^{2+}-ATPase.

8. The fraction of ATPase activity that is vanadate-sensitive increases from approx 50% in detergent solubilized membranes to greater than 98% in the final product.
9. Avoid incubating gel samples at higher temperatures. As with other membrane proteins, boiling gel samples in SDS may induce irreversible aggregation of the ATPase.

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Reconstitution and Assay of Biogenic Membrane-Derived Phospholipid Flippase Activity in Proteoliposomes

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1. Introduction

Newly synthesized phospholipids in biogenic (self-synthesizing) membranes, such as the eukaryotic endoplasmic reticulum (ER) and bacterial cytoplasmic membrane (bCM), are initially located in the cytoplasmic leaflet of the membrane bilayer. In order to populate the exoplasmic leaflet of the bilayer to allow uniform bilayer growth, phospholipids have to be translocated (flipped) to the opposite membrane leaflet. Flipping does not occur spontaneously; in artificial bilayers, liposomal systems, and certain biomembranes, transverse movement of phospholipids, i.e., spontaneous transfer of a phospholipid from one side of the membrane to the other, occurs only very slowly—if at all. Nevertheless, it is clear from a number of studies that transbilayer movement of phospholipids occurs rapidly in the ER and bCM by a bidirectional, diffusion process facilitated by specific membrane proteins and requiring no metabolic energy. The latter observation largely rules out conventional activities of the ABC family of transporters, which are involved in energy-coupled, vectorial transport of solutes and some phospholipids. Although certain α-helical membrane-spanning peptides appear able to promote transbilayer movement of some classes of phospholipid in synthetic membranes ***(1,2)***, it is generally hypothesized that specific proteins, called flippases, are required for phospholipid flipping in the ER and bCM ***(1,3–9)***.

Biogenic membrane phospholipid flippases have yet to be identified. This chapter deals with recently described protocols to reconstitute flippase activity in proteoliposomes generated from detergent extracts of ER or bCM, as well as

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procedures to measure phospholipid flip-flop in the reconstituted vesicles ***(4–8)***. Briefly, ER or bCM preparations are solubilized in Triton X-100, and a clear detergent extract is prepared by eliminating insoluble material by ultracentrifugation. The Triton extract is supplemented with egg phosphatidylcholine and trace amounts of a labeled reporter phospholipid, before being treated with detergent adsorbing beads. Gradual removal of detergent promotes the formation of unilamellar proteoliposomes with diameters in the size-range 150–300 nm. These vesicles are then assayed for their ability to flip the labeled reporter phospholipid across the bilayer.

By varying the amount of Triton extract included in the reconstitution, it is possible to generate proteoliposomes containing, on average, a single functional flippase per vesicle. Increasing the amount of Triton extract beyond the amount required to populate each vesicle with a single flippase results, as expected, in an increase in the rate of flipping ***(7);*** decreasing the amount of Triton extract results in fewer flippase-equipped vesicles, and consequently a reduction in transport amplitude with no alteration in transport rate. The protein:phospholipid ratio of proteoliposome preparations containing an average of one functional flippase per vesicle provides an excellent basis to estimate the relative abundance of active flippases present in the crude Triton extract. Given the average size of the vesicles (determined by dynamic light scattering or other means) and based on assumptions concerning the average molecular mass of biogenic membrane proteins (approx 50 kDa is a good estimate (*see* **ref. *4***), thickness of the membrane bilayer (approx 4 nm), the cross-sectional area of a phospholipid (approx 0.7 nm^2), and the number of phospholipid molecules per vesicle (approx 550,000 phospholipid molecules per 250 nm diameter vesicle), it is possible to calculate the abundance of functional flippases. We have determined this to be approx 0.2% by weight of biogenic membrane proteins (the number is similar for both rat liver ER and *Bacillus subtilis [B. subtilis]* cytoplasmic membrane ***[4,5,7]***); estimates as high as 0.6% have been reported for rat liver ER ***(9)***. The reconstitution protocol and activity assay have been used to enrich for functional flippases and can be used to isolate, identify, and characterize the protein. In purification protocols we reconstitute at a low protein:phospholipid ratio such that not all vesicles contain a flippase. Under these conditions, an increase in the protein:phospholipid ratio for a protein fraction produces a proportional increase in the extent of transport (because more vesicles are flippase-competent at the higher protein:phospholipid ratio). Such data can be used to generate a traditional specific activity measure (transport amplitude normalized to the protein:phospholipid ratio) that can, in turn, be used to guide a purification effort (*see* **refs. *4–8*** for further details).

The assay to measure flippase activity makes use of [^{3}H]dipalmitoylphosphatidycholine ([^{3}H]DPPC) as a transport reporter, and relies on the

ability of phospholipase A_2 to hydrolyze only those phospholipids present in the outer leaflet of liposomes and proteoliposomes without compromising the intactness of the vesicle membrane ***(7)***. Thus, in protein-free liposomes or proteoliposomes lacking a flippase, only 50–60% of the [^{3}H]DPPC will be hydrolyzed, corresponding to the outer leaflet pool. However, for proteoliposomes equipped with a flippase, [^{3}H]DPPC located in the inner leaflet will be flipped out and hydrolyzed as well (phospholipids and hydrolysis products will be transported from the outer leaflet to the inner leaflet to compensate), resulting in a predicted hydrolysis of 100%. Although the extent of phospholipase A_2-mediated hydrolysis is considerable, the simultaneous presence of the hydrolysis products, lyso phospholipid and fatty acid, preserves the membrane permeability barrier. [^{3}H]DPPC resembles natural phospholipids and is commercially available, making it an attractive choice for transport reporter. The procedure we describe is easily adapted to other measures of flippase activity, for instance using fluorescent NBD-phospholipids or other phospholipid analogs. Examples of assays using other phospholipid reporters may be found in ***(4–6)***, and ***(8–9)***

2. Materials

2.1. Solubilization of Biogenic Membrane Proteins

1. Biogenic membrane preparation, e.g., rat liver endoplasmic reticulum vesicles, bacterial cytoplasmic membrane vesicles (procedures for the preparation of these membranes may be found in ***[4–9]***). The protein concentration of the membrane suspension is typically in the range 5–25 mg/mL. The membranes are typically flash-frozen in liquid nitrogen after preparation and stored at −80°C.
2. Buffer A1: 1 *M* potassium acetate, 10 m*M* HEPES pH 7.5.
3. Buffer A2: 0.5 *M* potassium acetate, 10 m*M* HEPES pH 7.5.
4. Buffer B: 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.
5. Buffer C: 2% Triton X-100 (w/v), 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.

2.2. Reconstitution of Liposomes and Proteoliposomes

1. Egg phosphatidylcholine (egg PC), 100 mg/mL in chloroform (stored at −20°C).
2. Triton X-100, 10% (w/v) solution. Triton X-100 that has been specially purified for membrane research is recommended. Store at 4°C.
3. A stock solution of 200 m*M* HEPES pH 7.5.
4. A stock solution of 2 *M* NaCl.
5. 13 × 100 mm Pyrex screw-cap tubes and caps.
6. SM-2 BioBeads, 20–50 mesh (BioRad, Hercules, CA).
7. Methanol.
8. Nitrogen or argon for drying.
9. 2 mL Dounce homogenizer.
10. [choline-methyl-^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC).

2.3. Flippase Assay

1. Buffer B: 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.
2. Liposomes or proteoliposomes, prepared as described in **Subheading 3.2.** containing [^{3}H]DPPC.
3. Perchloric acid, ammonium molybdate and ascorbic acid for phosphate assay.
4. Phospholipase A_2, from *Naja naja* venom (obtained as a powder from Sigma; dissolved in water at a concentration of 10,000 U/mL in water, and stored in aliquots at –20°C).
5. 100 m*M* $CaCl_2$.
6. 2 *M* NaCl.
7. 120 m*M* ethylene glycol-*bis* (β-aminoethyl ehter) *N,N,N',N',*-tetraacetic acid (EGTA) (pH 7.0).
8. Chloroform and methanol for extraction.
9. Thin layer chromatography (TLC) solvent, freshly prepared at time of use: chloroform:methanol:28% ammonia (65:25:5, by volume).
10. Silica 60 TLC plates, activated by heating in a 100°C oven for at least 1 h.

3. Methods

3.1. Solubilization of Biogenic Membrane Proteins Including the Flippase

1. Wash membranes with high salt to remove peripheral membrane proteins; make sure membranes are fully suspended by using a Dounce homogenizer (*see* **Note 1**). Add an equal volume of Buffer A1. Mix well and incubate on ice for 30 min. Centrifuge to pellet the membranes (175,000*g*, 30 min, 4°C). Remove the supernatant and wash the pellet with ice-cold Buffer A2.
2. Extraction of integral membrane proteins (*see* **Notes 2** and **3**): Resuspend the membranes in Buffer B, by homogenizing in a total volume comparable to that of the starting membrane preparation. Add an equal volume of Buffer C. Mix well and incubate on ice for approx 30 min. The mixture should be less turbid than the starting material. Spin the sample in an ultracentrifuge at 175,000 *g* for 30 min, 4°C.
3. Carefully remove the supernatant (Triton Extract or TE) without disturbing the fluffy surface of the pellet. The TE typically contains 50–65% of the protein in the salt-washed membranes (*see* **Note 4**).

3.2. Reconstitution of Liposomes and Proteoliposomes

1. The procedure described is for the reconstitution of vesicles from a 1-mL sample containing approx 4.5 μmol egg phosphatidylcholine with or without Triton Extract (to prepare proteoliposomes or liposomes, respectively) in 10 m*M* HEPES pH 7.5, 100 m*M* NaCl, 1% (w/v) Triton X-100. We routinely scale this procedure to 2 mL samples by doubling all the reagents but processing all samples for the same length of time as for the 1-mL reconstitution. The procedure is adapted from Lévy et al. ***(9)***.
2. Wash the SM2 Bio-Beads (weigh out more than you need, as beads are invariably lost during the washing protocol) successively with methanol (twice); water (three

times); then 10 m*M* HEPES pH 7.5, 100 m*M* NaCl (once). Use wash volumes of 25 mL/*g* beads. A 1-mL reconstitution procedure requires 300 mg of wet beads: 100 and 200 mg aliquots of wet washed beads may be prepared ahead of time and stored, respectively, in capped 1.5 mL Eppendorf tubes. Make sure that the caps are tightly closed so that beads remain moist. (*see* **Note 5**).

3. Dry 32 μL 100 mg/mL egg PC (approx 4.5 μmol) in a screw-cap 13 × 100-mm glass tube under a stream of nitrogen (*see* **Note 6**). Use a Hamilton syringe specifically set aside for this purpose—avoid plastic-ware.
4. Add water, Triton X-100, HEPES buffer, and NaCl to the dried lipid such that the final volume and concentration after the addition of protein sample will be 1 mL at 1% Triton X-100, 10 m*M* HEPES, 100 m*M* NaCl. Before adding protein (Triton Extract), vortex every few minutes until the lipid is dissolved—this takes about 15 to 20 min at room temperature. The solution should be completely clear. Bubbles/froth may be cleared by centrifuging the sample briefly in a table-top clinical centrifuge (e.g., 1000*g*, 5 min), leaving the tube uncapped. (*see* **Note 7**).
5. Add the TE. Vortex gently to mix, but avoid generating froth. For liposomes, omit TE. The total volume of the sample should be 1 mL.
6. Add ~100 mg of wet washed beads. Reserve the ~200 mg aliquot of beads for the next step. Incubate at room temperature on an end-over-end mixer (*see* **Note 8)** for 3–4 h. The mixture should become slightly turbid.
7. Add the 200-mg aliquot of wet beads. Transfer the end-over-end mixer to 4°C and continue mixing for at least 12 h. This step is usually done overnight, so that the proteoliposomes are ready for the flippase activity assay the following morning.
8. Remove the turbid supernatant from the beads (rinse the beads, if desired, with a small volume of 10 m*M* HEPES pH 7.5, 100 m*M* NaCl, then add the rinse to the supernatant) and centrifuge at 265,000*g* for 45 min in a TL-100 ultracentrifuge (Beckman) to pellet the vesicles. Remove the supernatant and resuspend the vesicles in 10 m*M* HEPES pH 7.5, 100 m*M* NaCl; centrifuge again, and resuspend the pelleted vesicles in a small volume (approx 400 μL) of the same buffer. It is important that the proteoliposome suspension appears uniform by eye; rather than pipet-mixing for this purpose, we recommend using a 2-mL Dounce homogenizer.
9. Protein recovery after reconstitution is typically approx 50%, phospholipid recovery ranges between 50–70%. Measurement of protein in the reconstituted vesicles is frequently compromised by the presence of phospholipid. To circumvent this problem, the protein is precipitated using a chloroform/methanol mixture, then dissolved in SDS (*see* **Note 9**).

3.3. Flippase Assay

1. Take a small aliquot (5–10 μL) of the liposome or proteoliposome sample to measure phospholipid content (*see* **ref.** ***[11]***). Based on this measurement, resuspend an aliquot of the vesicles at a concentration of 1 m*M* (lipid phosphorus) in Buffer B (*see* **Notes 10** and **11**).

2. Setup reactions in Eppendorf tubes on ice. Each Eppendorf tube should contain: 21 μL Buffer B, 1.5 μL 100 m*M* $CaCl_2$; 1.5 μL 2 *M* NaCl; 3 μL membranes (1 m*M* lipid phosphorus).
3. Preincubate at 30°C for 1 min.
4. Start the assay by adding 3 μL phospholipase A_2 (10,000 U/mL). Vortex briefly to mix. The total reaction volume is 30 μL. Incubate at 30°C; hydrolysis is complete in approx 15 min (*see* **Note 12**).
5. At desired times, stop hydrolysis by adding 30 μL 120 m*M* EGTA and 165 μL water (note that phospholipase A_2 activity is Ca^{2+}-dependent).
6. Extract lipids: Add 250 μL chloroform and 250 μL methanol, then vortex to mix and centrifuge to get a clear separation of phases (a few minutes in a microfuge). Save the lower phase. Remove the upper phase and transfer to a fresh eppendorf tube. Add 250 μL mock lower phase, vortex, and centrifuge. Collect the lower (organic) phase and pool with the original lower phase. Wash the pooled lower phase with mock upper phase. Collect the lower phase, dry under nitrogen, and dissolve in 40 μL chloroform:methanol (1:1, v/v).
7. TLC analysis: Spot the lipid extract on an activated Silica 60 TLC plate. Develop in freshly prepared chloroform:methanol:28% ammonia (65:25:5, by volume). Air-dry the plate, and visualize the distribution of radioactivity on the plate with a TLC scanner, phosphorimager, or fluorography followed by densitometry of the spots. Rf values for lyso PC and PC in this solvent system are approx 0.1–0.2 and approx 0.3–0.4, respectively.
8. The extent of hydrolysis is calculated as [(lyso [^{3}H]PC)/(lyso [^{3}H]PC + [^{3}H] DPPC)].
9. Roughly 45–50% hydrolysis can be expected in the case of liposomes (i.e., % hydrolysis of phospholipids in the outer leaflet of the vesicles). For proteoliposomes, % hydrolysis ranges from 50–75% depending on the activity of the proteins and the ratio of protein/phospholipid in the reconstituted vesicles (*see* **Note 13**).

4. Notes

1. Resuspension after each centrifugation step should be done using a Dounce homogenizer. Repeated pipeting is not adequate to break up small membrane clumps. For example, 10 or 20 passes with the Dounce helps to minimize clumping and maximize the membrane surface available for detergent extraction when preparing the Triton extract, or for phospholipase A_2-catalyzed hydrolysis during the flippase activity assay.
2. In this protocol, we specify Triton X-100 because we know it to be compatible with the flippase activity assays we have tested. Liposomes and proteoliposomes prepared using extracts from other detergents may be compatible with many downstream applications, but some detergents cause artifactual flip-flop of phospholipids in liposomes (unpublished results).
3. After solubilization of flippase activity in 100 m*M* NaCl, 1 % (w/v) Triton X-100, it is possible to lower both salt and detergent concentration 10-fold to facilitate

chromatography without causing any apparent decrease in the amount of soluble flippase activity.

4. The Triton extract may be frozen (–20°C) for storage without loss of flippase activity.
5. SM2 BioBeads should be washed freshly for each reconstitution. It is possible to keep them in buffer at 4°C for a few days, but prolonged storage of washed beads leads to poor performance.
6. Lipid tracers (e.g., [^{3}H]DPPC) should be dried from solution along with the egg PC. These will be incorporated into both leaflets of the phospholipid bilayer.
7. It is sometimes desirable to reconstitute larger volumes of a sample. This method has been used in instances where almost all of the final reconstitution volume came from the protein sample. For example, the lipids may be dissolved in 82 μL of 10% Triton X-100, then brought to 1 mL by adding 918 μL of a protein sample in 0.2% Triton X-100, 10 m*M* HEPES, 100 m*M* NaCl.
8. Mixing end-over-end is essential to remove all the detergent. A platform rocker may be used instead of an end-over-end mixer, but the mechanics of mixing are different—we have had more consistent success with end-over-end mixing.
9. To determine the protein/phospholipid ratio of the proteoliposomes, the protein is precipitated from 100 μL of proteoliposomes by adding 375 μL chloroform/methanol 1:2 (v/v) in a 1.5-mL Eppendorf tube. After centrifugation in a microcentrifuge for 10 min, the supernatant is transferred to a glass test tube. The pellet is washed with another 300–500 μL chloroform/methanol 1:2 (v/v), and the supernatants are pooled, dried, and may be used for a lipid phosphorus determination (*see* **Note 10** and ***[11]***). To quantify protein, the pellet is dried and dissolved overnight at room temperature in 100 μL 1% sodium dodecyl sulfate (SDS). The redissolved protein can be assayed by the micro-BCA method (Pierce Chemical Co.), which is tolerant of 0.1 % SDS and <1 % Triton X-100. We have also successfully used a different assay procedure (***12***) that does not require prior precipitation of protein.
10. Accurate determination of lipid phosphate before phospholipase A_2 treatment is crucial to the performance of the assay (*see* **Note 2**). Phosphate determination by the method of Rouser et al. ***(7)*** takes about 90 min, from start to finish. Briefly, phospholipids are extracted with a chloroform:methanol mixture, the extract is dried, hydrolyzed with perchloric acid to release inorganic phosphate from the glycerophospholipid molecules, and the released phosphate is assayed in a color reaction using an ascorbic acid-ammonium molybdate mixture.
11. The ratio of phospholipase A_2 to the number of vesicles is important. The proportions given here result in an average of at least 1 phospholipase molecule/vesicle. A lower ratio does not allow for hydrolysis of all vesicles, because the phospholipase A_2 tends to associate with vesicles and "hops" only infrequently between them.
12. For kinetic studies of flipping activity, the incubation time can be varied. Phospholipids in the outer leaflet of the vesicle are hydrolyzed within one minute ($t_{1/2}$ approx 0.1 min ***[7]***), and the entire accessible DPPC pool in flippase-containing proteoliposomes is hydrolyzed within 15 min.
13. We found that vesicles maintain their integrity during phospholipase treatment. This was tested by monitoring the leakage of fluorescent FITC-Dextran (4300 Da).

The Dextran was trapped in the vesicles at a self-quenching concentration (the dextran was added at a sufficiently high concentration prior to the addition of SM2 Bio-Beads for reconstitution, resulting in a proportion of the dextran being trapped within the lumen of the vesicles). The dilution of the FITC-dextran accompanying release from the vesicles could be monitored by an increase in fluorescence. Treatment of proteoliposomes with phospholipase A_2 for 20 min at 30°C resulted in a low level of leakge (5%, compared with the signal obtained when the vesicles were disrupted with detergent) (*see* **ref. 7**).

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23

Expression, Purification, and Reconstitution of Rat Liver Carnitine Palmitoyltransferase I

Nicholas F. Brown

1. Introduction

1.1. The Mitochondrial Carnitine Palmitoyltransferase (CPT) System.

The mitochondrial carnitine palmitoyltransferase (CPT) system of enzymes permits the entry of long-chain acyl groups into the mitochondrial matrix, the site of β-oxidation. First conceptualized as a mechanism for mitochondrial fatty acid transport almost four decades ago ***(1,2)***, this multicomponent enzyme/transporter system is now recognized to play a primary role in the regulation of mitochondrial fatty acid oxidation and, hence, constitutes a critical control point in cellular lipid metabolism ***(3–5)***.

Whether derived from the plasma or from *de novo* synthesis in lipogenic cells, cellular fatty acyl groups exist primarily as CoA esters. CPT I, the first component of the CPT system, is located on the outer mitochondrial membrane and catalyzes the transfer of a long-chain acyl group from CoA to carnitine (*see* **Fig. 1**). Whereas the outer mitochondrial membrane is freely permeable to CoA and carnitine esters, the inner membrane presents a barrier and a specific carnitine/acylcarnitine carrier (CAC) allows the acyl-carnitine formed by the CPT I reaction to cross into the mitochondrial matrix. A distinct gene product, the enzyme CPT II, then reverses the CPT I reaction, regenerating acyl-CoA and releasing carnitine (which exits the matrix via the CAC). The acyl-CoA formed can enter the pathway of β-oxidation to provide cellular energy or, in the liver, serve as the substrate for ketone body synthesis ***(3–5)***.

Regulation of the CPT system is invested primarily at the level of CPT I, which is potently inhibited by malonyl-CoA, the product of the acetyl-CoA car-

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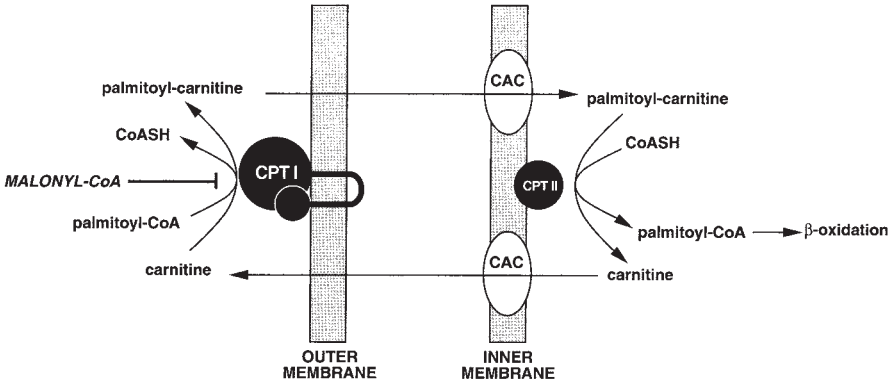

Fig. 1. The mitochondrial carnitine palmitoyltransferase system. CPT I and CPT II, carnitine palmitoyltransferases I and II; CAC, carnitine/acylcarnitine carrier.

boxylase (ACC) reaction, and the first committed intermediate in the opposing pathway of *de novo* fatty acid synthesis. First conceived in the context of hepatic metabolism, this elegant regulatory mechanism mediates the reciprocal regulation of *de novo* fatty acid synthesis and β-oxidation, avoiding a futile cycle ***(6)***.

However, in recent years, it has become apparent that the same regulatory mechanism also operates in tissues where fatty acid synthesis does not occur, such as skeletal muscle and heart ***(7–11)***. These tissues contain ACC activity and the level of malonyl-CoA is seen to fluctuate with metabolic state as in the liver. It is believed that in these tissues, ACC and malonyl-CoA are present purely as regulators of CPT I.

Although CPT II and the CAC exist as the same proteins in all cells, CPT I is present as two isoforms, the products of separate genes ***(4)***. These have been designated L-CPT I (or liver type) and M-CPT I (or muscle type) in accordance with the tissues in which they were originally described, although it has been shown that each is present in a variety of tissues and cell types ***(4,12)***. Although 63% identical at the amino acid level, the two isoforms of CPT I display radically different kinetics with regard to inhibition by malonyl-CoA (IC_{50} of 3–10 μ*M* vs 0.03 μ*M*, for L- and M-CPT I, respectively) and in terms of the *Km* for the substrate carnitine (30 μ*M* vs 500 μ*M*). Furthermore, in the liver, the sensitivity of L-CPT I to inhibition by malonyl-CoA is seen to change with (patho)physiological state, becoming desensitized on fasting and in experimentally induced diabetes ***(13–15)***. This mode of regulation may be mediated by changes in the mitochondrial membrane lipids ***(15–17)***.

Expression and analysis of recombinant native and mutated forms of the CPT enzymes has begun to shed some light on how the enzymes function and are regulated at a molecular level. A model for the topology of CPT I in the outer mitochondrial membrane has been developed ***(18)*** and we are beginning to understand how certain regions of the molecule contribute to function ***(19–25)***. As presented in **Fig. 1**, CPT I is believed to be comprised of two primary domains, both lying on the cytosolic aspect of the membrane and linked by two membrane spans and a loop of about 25 amino acids in the intermembrane space. The larger, C-terminal domain (approx 650 amino acids) contains the catalytic center of the protein ***(19)***. The approx 50 amino acid N-terminal domain is necessary for inhibition by malonyl-CoA ***(19–21)***.

Despite these advances, the nature of CPT I as an integral membrane protein has placed significant hurdles in the path of those who would study the enzyme. In particular, CPT I loses catalytic function on removal from the mitochondrial membrane with detergents ***(26)***, precluding preparation of a pure and active enzyme sample by conventional means. We have recently overcome this obstacle by combining recombinant L-CPT I (purified in detergent solution) with phospholipids to generate proteoliposomes in which active and malonyl-CoA sensitive enzyme is regained ***(17)***. This purified, active preparation will facilitate further study into the molecular regulation of L-CPT I. In particular, the reconstituted material is well suited to examine the relationship between the catalytic and regulatory domains of the enzyme and how malonyl-CoA sensitivity is influenced by the phospholipid milieu.

*1.2. Pichia pastoris (*P. pastoris*) as an Expression System*

In this chapter, we describe in detail our methods for the expression, purification, and reconstitution of recombinant rat L-CPT I. Because this volume is primarily intended to describe methods for the analysis of membrane proteins, we have concentrated on the growth of yeast cells expressing L- CPT I and on the purification and reconstitution of the enzyme. However, we also provide an explanation of how the expression plasmid was designed and manufactured and how a line of *P. pastoris* cells expressing rat L-CPT I was generated (**Subheading 3.2.**).

We chose to express L-CPT I in the yeast, *P. pastoris*, that has been increasingly exploited to overexpress functional proteins ***(27–31)***. As a yeast, *P. pastoris* has all the advantages for protein expression of its more familiar cousin *S. cerevisiae*. First, it can be manipulated easily on a small scale for generation and analysis of recombinant clones and culture is readily scaled up to grow large amounts of selected high-expressing strains. Second, because they are

eukaryotic cells, they possess much of the machinery required for the correct folding, intracellular targeting, and membrane insertion of exogenous membrane proteins. Third, yeast have no endogenous CPT activity ***(17,19)***. In addition, using the pPICZ series of plasmids from Invitrogen, transformation of *P. pastoris* cells results in stable, chromosomal integration of the expression cassette, which means that after initial selection of transformants, culture can be performed in the absence of antibiotic selective pressure, an important cost consideration for large cultures. We ***(17)*** and other groups ***(21,22,32)*** have found *P. pastoris* to be a highly successful system for expression of CPT enzymes. Our own experience was that we achieved 200-fold better expression than we had previously obtained in *S. cerevisiae* ***(17,19)***.

1.3. Overview of the Experimental Procedures

Cultures of *P. pastoris* transformed with the L-CPT I expression construct are grown for 24 h in the presence of MeOH, which induces expression of the exogenous cDNA (*see* **Subheading 3.**). Spheroplasts are prepared by enzymatic removal of the cell wall. These are then broken mechanically and a membrane fraction containing the recombinant L-CPT I is prepared. The detergent, Triton X-100, is used to extract L-CPT I from the membranes.

To facilitate purification of the recombinant protein, we expressed rat L-CPT I with six histidine residues (his6) fused to the C-terminus of the enzyme. This permits a relatively facile purification due to the high affinity of this tag for a Ni-containing agarose matrix. Now a standard procedure, Ni-affinity purification of his6 tagged proteins has the unusual advantage that it is effective even under conditions where the protein is denatured. In the case of L-CPT I, this allows purification in Triton X-100 solution, conditions that inactivate the enzyme and make conventional purification procedures difficult to apply. The presence of the tag does not interfere with catalysis or affect the kinetic properties of the enzyme ***(17)***. The typical yield of purified L-CPT I is approx 2 mg/L original culture.

For the reconstitution, phospholipids are dissolved in aqueous solution using the detergent octylPOE. These are combined with a concentrated preparation of L-CPT I in Triton X-100 and removal of the detergents is accomplished using the adsorbant, BiobeadsSM2. Under these conditions the phospholipids and protein combine to form proteoliposomes which contain active and malonyl-CoA sensitive L-CPT I.

A detailed method for assay of CPT activity is also provided. This assay is suitable for samples prepared from mammalian tissues as well as the yeast fractions and reconstituted L-CPT I obtained during the procedures provided here (*see* **Notes 1** and **2**).

2. Materials

2.1. CPT Assay

2.1.1. Stock Solutions and Reagents

1. Palmitoyl-CoA (Sigma, P-9716): Dissolve in water at 20 m*M*. The stock solution can be stored at −20°C for several weeks and is stable upon freeze/thaw.
2. L-carnitine (Sigma, C-0283): Stock solution is 100 m*M* in water. Store at −20°C
3. L-[N-methyl-^{14}C]carnitineHCl is obtained from American Radiolabeled Chemicals (ARC 308) at 0.1 mCi/ml in 50 % EtOH and stored at 4°C.
4. 150 m*M* KCl, 5 m*M* Tris-HCl, pH 7.2. Adjust pH with HCl and store at 4°C.
5. Malonyl-CoA (Sigma, M-4263). Prepare a stock solution at 10 m*M* in 150 m*M* KCl/ 5 m*M* Tris-HCl, pH 7.2 and store at −20°C. The stock can be kept for several weeks and is stable upon freeze/thawing. A working solution is prepared by dilution in the same buffer to 1 m*M*.
6. 4 *M* $MgCl_2$: Stable at room temperature.
7. Rotenone (Sigma,R-8875): Stock solution is 40 mg/mL in acetone. Store in a tightly capped tube at 4°C. Discard after 1 mo.
8. 210 m*M* Tris-HCl, pH 7.2: Adjust pH with HCl. Store at 4°C.
9. 1-butanol.
10. Solid reagents: bovine serum albumin, essentially fatty acid free (Sigma, A-6003)
11. Adenosine triphosphate (ATP) (Sigma, A-2383).
12. KCN (Aldrich, 20,781-0). Note, KCN is highly toxic and releases hydrogen cyanide gas when in contact with acid. Gloves should be worn when handling KCN and caution should be observed when storing.
13. Substrate Mix (5×): To 4.75 ml water, add 62.5 μL 20 m*M* palmitoyl-CoA, 125 μL unlabeled 100 m*M* L-carnitine, and 62.5 μL [^{14}C]carnitine. Final concentrations in the reaction are 50 μ*M* palmitoyl-CoA, 500 μ*M* carnitine and 0.25 μCi/mL [^{14}C]carnitine (*see* **Note 3**). Substrate Mix can be stored at −20°C for several weeks and can be frozen and thawed several times without harm (*see* **Note 4**).
14. Assay Cocktail (2×): 210 m*M* Tris-HCl (pH 7.2), 2 % (w/v) bovine serum albumin, 8 m*M* ATP, 8 m*M* $MgCl_2$, 0.5 m*M* glutathione (reduced form), 80 μg/mL (w/v) rotenone and 4 m*M* KCN (*see* **Note 5**). To prepare the assay cocktail, dissolve the bovine serum albumin (BSA) in the required volume of Tris-HCl, followed by the ATP, glutathione, and KCN (as solids), followed by the $MgCl_2$ (from the 4 *M* stock) and finally the rotenone (from the 40 mg/mL stock in acetone). Note, the buffer will turn cloudy on addition of the rotenone, but will clear on continued stirring. Cocktail must be prepared fresh daily.

2.2. Expression of L-CPT I *in* P. pastoris

2.2.1. EasySelect Pichia Expression Kit

The EasySelect *Pichia* Expression Kit from Invitrogen provides the plasmid vectors, *P. pastoris* strains and reagents for *Pichia* transformation. The complete

components of the kit are available in their catalogue or web site (*see* **Note 6**). We list below only those components used for expression of L-CPT I.

1. Plasmid vector, pPICZ A. This vector contains the Zeocin antibiotic resistance gene and the AOX1 promoter to drive expression of the exogenous cDNA when the yeast are grown with MeOH as sole carbon source. Immediately 3' to the site of cDNA insertion (polylinker) are bases encoding a c-*myc* epitope and six histidine residues (his6) which can be expressed with the cDNA as part of a fusion protein. However, these additional vector sequences were not used in the expression of rat L-CPT I.
2. Yeast strain, GS115 (Mut+, His+). This strain of *P. pastoris* must be grown in histidine containing medium.
3. EasyComp *Pichia* Transformation Kit. The reagents supplied allow a facile transformation of *P. pastoris* with linearized plasmid DNA, which results in chromosomal integration of the recombinant DNA.
4. Antibiotic, Zeocin. The Zeocin antibiotic resistance gene on the pPICZ series of plasmids is expressed in both *Escherichia coli* (*E. coli*) and *P.pastoris*. Consequently, Zeocin resistance can be used for selection during manufacture of the expression construct in *E. coli* and also for selection of *P. pastoris* transformants.

2.2.2. Manufacture of Expression Construct

1. Polyacrylamide gel electrophoresis (PAGE)-purified oligonucleotides were obtained from Integrated DNA Technologies, Inc., IO.
2. Enzymes for standard molecular biology procedures were from: New England Biolabs (T4 DNA ligase, Vent Polymerase for polymerase chain reaction (PCR), calf intestinal phosphatase, *Pme I*) and Boehringer Mannheim (*Sfu I*).
3. Qiagen Plasmid Midi Kit (Qiagen), (*see* **Note 7**).

2.3. Growth and Propagation of P. pastoris Expressing L-CPT I

2.3.1. Stock Solutions for Culture Media

1. 10 × GY: Add 50 mL glycerol to 450 mL water. Autoclave 30 min. Stable for months stored at 4°C.
2. 10 × YNB: Dissolve 53.6 g yeast nitrogen base without amino acids (with ammonium sulfate, Difco 291940) in 400 mL water. Filter sterilize. Stable for 1 mo at 4°C.
3. 500 × B: Dissolve 20 mg biotin (Sigma, B-4501) in 100 mL water. Filter sterilize. Stable for months at 4°C.
4. 100 × H: Dissolve 400 mg histidine (Sigma, H-8000) in 100 mL water, heating if necessary. Filter sterilize. Stable for months at 4°C.

2.3.2. Culture Media

1. Making liquid culture media: For small (25 mL) cultures, autoclave the indicated volumes of water in a 500-mL bottle and then add the other presterilized components of the medium as described below. To initiate culture, add 25 mL to a sterile,

baffled 125 mL flask, and then inoculate as appropriate (*see* **Note 8**). The remaining medium is stable for at least 1 mo at 4°C. For large (400 mL) cultures, autoclave water in a 2-L baffled flask, and then add the other components directly to the flask. The culture volume should never exceed 20 % of the capacity of the flask.

2. MGYH (minimal glycerol medium with histidine): Stock medium for small cultures; 200 mL autoclaved water, 25 mL 10 × GY, 25 mL 10 × YNB, 0.5 mL 500 × B, 2.5 mL 100 × H. Large culture; 320 mL autoclaved water, 40 mL 10 × GY, 40 mL 10 × YNB, 0.8 mL 500 × B, 4 mL 100 × H.
3. Minimal methanol medium with histidine (MMH): Stock medium for small cultures: 225 mL autoclaved water, 25 mL 10 × YNB, 0.5 mL 500 × B, 2.5 mL 100 × H, 2.5 mL MeOH. Large culture; 350 mL autoclaved water, 40 mL 10 × YNB, 0.8 mL 500 × B, 4 mL 100 × H, 4 mL MeOH.

2.3.3. Culture Plates

1. YPDS plates/Z100 (yeast peptone dextrose sorbitol with 100 μg/mL Zeocin): These plates are used for initial selection of *P. pastoris* transformants. Dissolve 2 g yeast extract (Difco, 212750), 4 g Bacto-peptone (Difco,211677), 4 g glucose, and 36.4 g sorbitol in 150 mL water. Adjust volume to 200 mL and then add 4 g Bacto-agar (Difco, 214010). Autoclave 20 min. (Note: prolonged autoclaving of glucose-containing media causes caramelization of the glucose, which slows growth of the cells.) Cool to less than 60°C and add 250 μL of 100 mg/mL Zeocin before pouring.
2. YPD and YPD/Z100 plates (yeast peptone dextrose ± 100 μg/mL Zeocin): YPD plates are suitable for maintaining *P. pastoris* cells for routine inoculation of liquid cultures. The addition of Zeocin is not necessary, but reduces the risk of bacterial contamination. To 200 mL water, add 2 g yeast extract, 4 g bacto-peptone, 4 g glucose, and 4 g agar. Autoclave 20 min. Cool to less than 60°C and add 250 μL of 100 mg/mL Zeocin before pouring, if desired.

2.4. Purification of Recombinant L-CPT I

2.4.1. Solutions and Reagents

1. 100 m*M* Tris-H_2SO_4: Adjust to pH 9.4 with sulfuric acid. Stable at 4°C.
2. 1 *M* dithiothreitol (DTT). Dissolve in water and store at −20°C.
3. Sorbitol Buffer: 1.2 *M* sorbitol, 50 m*M* potassium phosphate, pH 7.4
4. DNAse I (Sigma, DN-25): Dissolve 10 mg of DNAse I in 1 mL water. Store at − 20°C in aliquots. Do not freeze/thaw repeatedly.
5. Phenylmethylsulfonylfluoride (PMSF): 100 m*M* in isopropanol. Vortex repeatedly until dissolved. Store at −20°C. The PMSF will come out of solution when stored and must be redissolved before use. PMSF containing solutions should be handled using gloves.
6. Mannitol Buffer: 0.6 *M* mannitol, 10 m*M* Tris-HCl, pH 7.4. Immediately before use, add PMSF from stock solution to 1 m*M*, while stirring vigorously. Some of the PMSF may come out of solution, forming a precipitate on the buffer surface. However, this will not affect the procedure.

7. 5 *M* NaCl: Store at room temperature.
8. 500 m*M* potassium phosphate, pH 8.0: Prepare by mixing 500 m*M* KH_2PO_4 and 500 m*M* K_2HPO_4 to pH 8.0. Store at room temperature.
9. 10% Triton X-100: To 90 mL water, add 10 mL Triton X-100. Store at 4°C.
10. 5 *M* imidazole (Sigma, I-0250): Store at room temperature.
11. Mercaptoethanol: Pure liquid is 14.3 *M*. Store at 4°C.

(Solutions 12–16 can conveniently be made from the stock solutions 7–10. Note: mercaptoethanol is added to a final concentration of 2 m*M* in the required volumes of Solutions 12–16 immediately before use.)

12. Extraction buffer: 500 m*M* NaCl, 50 m*M* potassium phosphate, pH 8.0. Store at 4°C.
13. Loading buffer: 500 m*M* NaCl, 50 m*M* potassium phosphate, pH 8.0, 1% (v/v) Triton X-100, 10 m*M* imidazole. Store at 4°C.
14. 1 *M* washing buffer: 1 *M* NaCl, 50 m*M* potassium phosphate, pH 8.0, 1% (v/v) Triton X-100, 10 m*M* imidazole. Store at 4°C.
15. 0.5 *M* washing buffer: 0.5 *M* NaCl, 50 m*M* potassium phosphate, pH 8.0, 0.1 % (v/v) Triton X-100, 10 m*M* imidazole. Store at 4°C.
16. Elution buffer: 0.5 *M* NaCl, 50 m*M* potassium phosphate, pH 8.0, 0.1 % (v/v) Triton X-100, 250 m*M* imidazole. Store at 4°C.
17. Lyticase (Sigma, L-4025): Store solid at −20°C.
18. Liquid nitrogen.
19. Ni-NTA Agarose (Qiagen). Store at 4°C.

2.4.2. Additional Items

1. Centricon-10 centifugal concentrators (Amicon).

2.5. Liposomal Reconstitution of L-CPT I

1. BiobeadsSM2 (Bio-Rad 152–8920): Stable indefinitely at room temperature. Biobeads are highly hygroscopic and will accumulate moisture with frequent opening of the container. However, we have not noted any problems associated with this.
2. 20 m*M* HEPES, pH 7.2: Adjust to pH 7.2 with HCl. Store at 4°C.
3. Asolectin (Sigma, P-3644): Store at −20°C. Asolectin is a complex mixture of phospholipids, primarily phosphatidylcholine, extracted from soy bean.
4. Solvents: Chloroform, diethyl ether. Open containers only in a fume hood.
5. n-Octylpolyoxyethylene (OctylPOE, Bachem, P-1140): Store at −20°C. The detergent solidifies upon storage and must be thawed prior to use.
6. Disposable spin columns. (Bio-Rad, 731–1550)
7. Nitrogen gas.

2.6. Removal of Detergents and Lipids for Protein Assay

1. Solvents: absolute ethanol, diethylether.
2. 10% (w/v) SDS. A disposable face-mask should be worn when weighing and dissolving solid SDS.

3. 10 *N* NaOH.
4. 5% SDS/0.1 N NaOH. Mix 4.9 mL water, 5 mL 10% SDS and 100 mL 10 *N* NaOH. Prepare fresh.
5. Bicinchoninic acid (BCA) Protein Assay Kit (Pierce).

2.7. Equipment

Most of the necessary equipment will be present in a well-equipped research laboratory (e.g., centrifuges [including ultracentrifuge], cold room, static water bath, 70°C freezer, rocking platforms). Additional items are the following:

1. Orbital incubator capable of accommodating up to 2 L flasks;
2. Oscillating 30°C water bath for CPT assay.

3. Methods

3.1. CPT Assay

Both CPT I and CPT II are routinely assayed in vitro in the direction of palmitoyl-carnitine formation, using [^{14}C]carnitine as substrate (*see* **Note 9**):

$$\text{Palmitoyl-CoA} + [^{14}\text{C}]\text{carnitine} \rightarrow \text{palmitoyl-}[^{14}\text{C}]\text{carnitine} + \text{CoASH}.$$

Assay tubes are prepared containing Substrate Mix, Assay Cocktail, and malonyl-CoA if desired. Reactions are started by the addition of the enzyme-containing sample and stopped by the addition of HCl. The labeled palmitoyl-carnitine product is extracted into 1-butanol.

1. Assays are performed in 12 × 75 mm plastic culture tubes. Prepare tubes in duplicate at room temperature by the addition of 100 µL substrate mix, 50 µL of 150 m*M* KCl/5 m*M* Tris, pH 7.2, and 250 µL of Assay Cocktail. Final concentrations of substrates during the reaction are 50 µ*M* palmitoyl-CoA and 500 µ*M* [^{14}C]carnitine. For assessment of malonyl-CoA inhibition, 50 µL of 1 m*M* malonyl-CoA is substituted for the KCl/Tris, giving 100 µ*M* in the assay.
2. Keep samples on ice until addition to the reaction. To start the reactions, add 100 µL of enzyme sample to the reaction tube (giving a final volume of 500 µL). Vortex briefly and immediately place in a 30°C shaking water bath. At the end of the incubation period, remove the tube from the water bath, add 500 µL of 1.2 *N* HCl and vortex. Begin reactions at timed intervals (1 tube every 15 s, with practice) and stop them in the same timed sequence. For blanks (duplicate tubes), add HCl and vortex *before* adding 100 µL of enzyme sample and vortexing again. After the reactions are terminated, the samples are stable and need not be processed immediately.
3. Add 500 µL 1-butanol and vortex each tube for 30 s. Centrifuge at room temperature in a swingout rotor at 1000*g* for 8 min. After the centrifugation, a white band of precipitated protein will be present between the lower, aqueous phase, and the

upper, butanol phase. Delay of more than a few minutes between vortexing the samples and centrifugation results in poor separation, with a thick protein band at the interface, which can interfere with subsequent steps. To avoid this, after all the tubes have been mixed for 30 s, vortex each sample again briefly, immediately prior to centrifugation.

4. Transfer 300 μL of the upper, butanol phase to a 1.5-mL microfuge tube containing 60 μL of water. Cap the tubes and vortex each for 15 s. Centrifuge at maximum speed in a microfuge at room temperature for 2 min.
5. Transfer 250 μL of the upper phase to a scintillation vial. Add 10 mL ScintiSafe 30% scintillant, cap, and shake thoroughly to mix (*see* **Note 10**). The samples should be allowed to rest for 1 h to obtain accurate counts.
6. Using the above protocol, the blank should appear as 200–300 cpm, and this value must be subtracted from the others. Typically, the reaction is linear up to approx 3000 cpm, but as with any enzyme assay, one must establish under each set of conditions how long the assay can be run before the reaction rate diminishes (*see* **Note 11**).

3.2. Expression of Rat L-CPT I in P. pastoris

3.2.1. P. pastoris *as a Nutrient-Regulated Expression System*

P. pastoris is a methylotrophic yeast. That is to say, it is capable of growth on MeOH as the sole source of carbon. In order to achieve this, the yeast can express two isoforms of the enzyme alcohol oxidase. One of these isoforms, the product of the *AOX1* gene, can accumulate to up to 30% of cellular protein. The *AOX1* gene promoter is induced by MeOH, but suppressed by glucose. It is this powerful promoter which is present in the pPICZ series of plasmids, and which drives expression of the exogenous cDNA when the cells are transferred to medium containing MeOH as sole carbon source (*see* **Note 6**).

As a consequence of this nutrient-regulated expression of the recombinant protein, it is necessary to culture the yeast cells in media containing various carbon sources at different stages of the project.

1. *Glucose in undefined medium (YPD or YPDS)*. For growth of untransformed cells, selection of transformants with Zeocin or maintenance of stock cultures, conventional medium is used, in which glucose serves as primary carbon source.
2. *Glycerol in defined medium (MGYH)*. Glycerol is used as carbon source during the growth of starter cultures for expression experiments. This provides for adequate growth rates, but does not present the problem of "carry-over" of glucose, when the cells are transferred into induction medium. The presence of low levels of glucose would inhibit expression from the *AOX1* promoter.
3. *MeOH in defined medium (MMH)*. When MeOH is the only carbon supply, the cells grow slowly, but expression of the exogenous cDNA is induced.

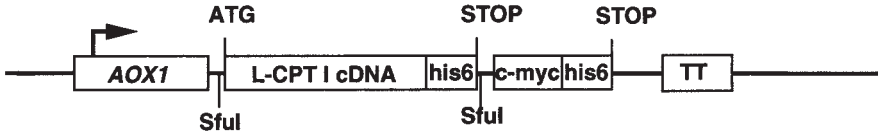

Fig. 2. Plasmid for expression of rat L-CPT I in *P. pastoris. AOX 1*, 942 bp fragment of the *AOX1* promoter capable of driving MeOH-inducible expression and which targets homologous recombination; ATG, initiator methionine; STOP, stop codon; his6, bases encoding 6 histidine residues; c-*myc*, bases encoding a c-*myc* epitope; TT, *AOX1* transcription termination region; *Sfu I*, restriction site for enzyme *Sfu I*.

3.2.2. The Expression Plasmid

Plasmid pPICZ-rL-CPT I-his6 is designed to express the full-length rat L-CPT I enzyme with the addition of six histidine residues at the C-terminus. A schematic of the final expression construct is shown in **Fig. 2**.

1. PCR was used to amplify the rat L-CPT I cDNA from a previously manufactured plasmid (pCMV6-rL-CPT I, **ref. *17***). The oligonucleotide primers were designed to modify the 5' and 3' ends of the coding region, as follows:

   5' oligonucleotide (CCC GAA TTC GAA ATA ATG GCA GAG GCT CAC CAA GCT): Encoded *EcoRI* and *SfuI* restriction sites, placed the initiator methionine codon in a preferred context (*see* **Notes 12** and **13**) and was complementary to the first 21 bases of the open reading frame.

   3' oligonucleotide (CCC GAA TTC GAA TTA ATG GTG ATG GTG ATG GTG CTT TTT AGA ATT GAT GGT GAG): Encoded *EcoRI* and *SfuI* restriction sites, a stop codon, six histidine residues and was complementary to the last 21 bases of the open reading frame.
2. The PCR product was digested with *SfuI* (removed the *EcoRI* sites, which were not used in this case) and the resulting major fragment gel purified.
3. Plasmid pPICZ A was restricted with *SfuI* and treated with calf intestinal phosphatase to prevent self-ligation of the plasmid.
4. The cut plasmid and insert were ligated using T4 DNA ligase and the product transformed into *E. coli* DH5α rendered competent with $CaCl_2$.
5. Transformed cells were plated on LB plates containing 25 μg/mL Zeocin.
6. Small cultures were inoculated from isolated colonies and plasmid DNA prepared according to standard procedures. Orientation of the insert was determined by restriction analysis of plasmid DNA minipreps.
7. One plasmid with the correctly oriented insert was subjected to sequencing of the entire insert by the dideoxy method (USB Sequenase II Sequencing Kit) to confirm the absence of errors introduced during the PCR reaction. The final plasmid was designated pPICZA-rL-CPT I-his6.

3.2.3. *Transformation of* P. pastoris *and Selection of a Strain for Further Study*

1. A large-scale preparation of pPICZA-rL-CPT I-his6 was accomplished from a 100-mL culture in LB with the Qiagen Midi-Prep Kit using a "100 tip" (yield 60 μg).
2. The plasmid (15 μg) was linearized by digestion with *Pmel* (*see* **Note 14**).
3. Cells of *P. pastoris* strain *GS115* were made competent and transformed with 6 μg of the linearized plasmid using the Invitrogen EasyComp *Pichia* Transformation Kit, according to the manufacturer's directions. The transformation mixture was plated on YPDS/Z100 and incubated for 2 d at 30°C. Approximately 80 colonies were obtained.
4. Eight colonies were used to inoculate liquid cultures in 25 mL MGYH (in 125-mL flasks) and grown, shaking for 48 h at 30°C.
5. The cultures were then centrifuged at 2000*g* for 10 min and the resulting pellets quantitatively resupended and used to inoculate 25 mL MMH, which was incubated, shaking, for a further 24 h at 30°C.
6. These cultures were then harvested and homogenized by a scaled down version of the detailed protocol given in **Subheading 3.4.1., step 2** and **Subheading 3.4.2., step 15**.
7. CPT assays were performed directly on these homogenates. Each of the strains analyzed had comparable CPT I activities. This strongly suggests that each contained a single copy of the endogenous cDNA (*see* **Note 15**). One of these strains was selected for further study.

3.3. Growth and Propagation of Large Cultures of P. pastoris Expressing Rat L-CPT I

1. Inoculate 25 mL MGYH in a 125-mL baffled flask from a single colony on a YPD/Z100 plate or with a "stab" of a frozen stock of *P. pastoris* expressing L-CPT I. Cover the flask with foil or a metal lid and grow with vigorous shaking at 30°C for 2–3 d (from a frozen stock) or overnight to 2 d (from a colony). At this time, the medium should be very cloudy. Transfer 5 μL of the culture to a microscope slide with a cover slip and examine under the microscope. Using a standard bench microscope (40× objective), *P. pastoris* cells will be clearly visible. Examine closely for the presence of bacterial contamination, which will be manifest as small dense particles. Because large scale *P. pastoris* culture is generally accomplished in the absence of antibiotic, it is important to confirm the absence of bacteria at every stage.
2. Take 0.1 mL of the culture and dilute with 1.9 mL water to read the absorbance at 600 nm. Multiply the absorbance read by 20 to obtain that of the original culture, which should be between 6 and 10 (*see* **Note 16**).
3. Centrifuge the culture at 2000*g* for 10 min and discard the supernatant.
4. Resuspend the pellet with 5 mL MGYH and transfer the entire culture to a 2-L baffled flask containing 400 mL MGYH. Cover the flask as above and grow with vigorous shaking at 30°C for 24 h.

5. Examine a portion of the culture under the microscope and read the absorbance (600 nm) of the culture as above. The absorbance of the undiluted culture should be between 12 and 15. Calculate the volume of culture equivalent to 400 absorbance units, as 400/absorbance of culture (for example, 33 mL for a culture with an absorbance of 12).
6. Transfer 6× this volume to 50 mL sterile tubes and centrifuge at 2000*g* for 10 min. Discard the supernatant.
7. Resuspend each of the six pellets with 5 mL MMH and transfer each to 400 mL MMH in six 2 L baffled flask. The initial absorbance of the cultures is now approx 1.0. Cover flasks with cheese-cloth squares held in place with a rubber band. When growing with MeOH as sole carbon source, it is critical that the cultures receive maximal oxygenation. Because MMH is a highly restrictive medium, the risk of bacterial contamination is very low at this stage. Grow with vigorous shaking at 30°C for 24 h.

3.4. Purification of Recombinant L-CPT I

3.4.1. Harvest of P. pastoris Cells Expressing Rat L-CPT I

1. Take 0.2 mL of the culture and dilute with 1.8 mL water to read the absorbance. Multiply by 10 to calculate the absorbance of the original culture which should be 6 to 8. Check for bacterial contamination.
2. Transfer the entire culture (2.4 L) to large centrifuge bottles and centrifuge at 2000*g* for 10 min.
3. Discard the supernatant. Resuspend the pellets with water and divide the total into three equal aliquots in preweighed 50 mL sterile tubes. Resuspension is achieved by repeatedly pipetting up and down with a 25-mL pipet. Make each up to a final volume of 40 mL with water.
4. Centrifuge at 2000*g* for 3 min at room temperature. Discard the supernatant, weigh the tubes, and calculate the wet weight of the pellet by the difference. Normal yields are 20–25 g, combined total weight.
5. Resuspend each with 40 mL water and centrifuge again, as earlier.

3.4.2. Removal of the Cell Wall and Preparation of a Membrane Fraction

1. Resuspend each pellet with 10 mL 100 m*M* Tris-H_2SO_4, pH 9.4, and add 250 µL of 1 *M* DTT.
2. Place in a 30°C water bath for 15 min. Invert to mix every 5 min.
3. Centrifuge at 500 *g* for 5 min at room temperature. After this spin, the pellet will be very loose (*see* **Note 17**). Carefully, pour off as much of the supernatant as possible, but it is not necessary to remove it all.
4. Gently resuspend the pellets, each to a final volume of 40 mL with sorbitol buffer.
5. Centrifuge as in **step 3** and discard the supernatant similarly.
6. Prepare enzyme mixture. To 3 mL sorbitol buffer add 60 mg of lyticase and 24 µL of 10 mg/mL DNAse I and mix. The lyticase may not dissolve completely, remaining partially as a fine suspension.

7. Gently resuspend each pellet of cells with 20 mL sorbitol buffer.
8. Take 10 μL from one of the tubes of cells and add to 1 mL water in a glass 12 × 75 mm culture tube. Set aside (*see* **step 11**).
9. To each tube of cells, add 1 mL enzyme mixture and invert thoroughly to mix.
10. Place in a 30°C water bath for 45 min, inverting every 15 min to mix.
11. Take 10 μL to 1 mL water, as in **step 8**. Vortex both aliquots and compare against a dark background. The post-enzyme sample should appear substantially clearer than the predigestion sample, due to osmotic disruption of the cells, which now lack a cell wall.
12. Centrifuge the cell suspensions as in **step 3** and discard the supernatant.
13. Resuspend with Sorbitol Buffer and centrifuge as in **step 3**.
14. Resuspend each of the three aliquots with 20 mL ice-cold mannitol buffer/1 m*M* PMSF. All subsequent steps are performed on ice or at 4°C.
15. Transfer to a glass homogenizer fitted with a tight pestle and homogenize with 20 cycles (i.e., up and down, 20 times).
16. Combine the samples and then divide into four equal aliquots in 30 mL ultracentrifuge bottles with screw caps.
17. Centrifuge at 100,000*g* for 30 min at 4°C.
18. Discard the supernatant. Recap the tubes and place in liquid nitrogen to freeze. Store the membrane pellets at −70°C. In this way, the pellets may be stored for several months before purification of L-CPT I.

3.4.3. Purification and Concentration of L-CPT I in Detergent Solution

1. Thaw one membrane pellet and add 20 mL ice-cold extraction buffer. Resuspend partially with a pipet and transfer to a glass homogenizer fitted with a tight pestle. Homogenize on ice with 20 cycles of the pestle.
2. Adjust the volume to 22.5 mL with Extraction Buffer and transfer to a 50-mL beaker with a stir bar. Add 2.5 mL 10 % Triton X-100 and stir ice-cold for 1 h.
3. Transfer to an ultracentrifuge bottle and centrifuge at 100,000*g* for 30 min.
4. Transfer the supernatant to a 50-mL beaker with a stir bar.
5. During the centrifugation, prepare the Ni-NTA agarose as follows. Resuspend the resin thoroughly, take 2 mL of the 50% slurry and centrifuge at 1000*g* for 5 min. Discard the supernatant, resuspend with 3 mL loading buffer and recentrifuge. Repeat the washing procedure and finally resuspend the resin with 1 mL of loading buffer (to regenerate 2 mL of a 50% slurry).
6. To the Triton X-100 supernatant, add 50 μL of 5 M imidazole (giving 10 m*M*) and the Ni-NTA agarose slurry. Stir gently ice-cold for 1 h.
7. Collect the Triton X-100 extract/Ni-NTA mixture and centrifuge at 1000*g* for 5 min in a 50-mL tube. Carefully remove the supernatant with a pipette.
8. Resuspend with 4 mL of loading buffer/2 m*M* mercaptoethanol, transfer to a 14-mL tube and centrifuge as earlier. Repeat this washing procedure two more times.
9. Resuspend the resin with 4 mL loading buffer/2 m*M* mercaptoethanol and transfer to a glass chromatography column (10 cm long × 1 cm diameter) with a glass frit

and fitted at the outlet with 3 cm of 1/8" internal diameter silicone rubber tubing on which a screw clamp has been placed. As the resin settles, use the clamp to adjust the flow rate to approx 0.2 mL min. Maintain this flow rate throughout the purification procedure.

10. Wash the column as follows:
    10 mL Loading Buffer/2 m*M* mercaptoethanol;
    20 mL 1 M Washing buffer/2 m*M* mercaptoethanol;
    3 mL 0.5 M Washing buffer/2 m*M* mercaptoethanol.
    Note: when changing buffers, allow the meniscus of the washing buffer to reach the resin, then add approx 0.3 mL of the next buffer. When this has entered the resin, add the remainder.
11. When the final washing buffer has all entered the resin bed, add 0.3 mL elution buffer/2 m*M* mercaptoethanol. When this has entered add a further 2.5 mL and collect the next 2.5 mL of effluent.
12. Transfer 2.0 mL of the collected effluent to a Centricon10 centrifugal concentrator and centrifuge at 4500*g* for 60 min in a fixed angle rotor (Sorvall SS-34). At this time, carefully measure the volume of the liquid remaining above the membrane using a pipet. If the volume is greater than 350–400 μL, centrifuge for a further 15 min.
13. Invert the concentrator and centrifuge at 750*g* for 2 min to collect the concentrated sample.

3.5. Liposomal Reconstitution of L-CPT I

3.5.1. Preparation of BioBeadsSM2

1. Weigh 1.0 g of Biobeads SM2 and place in a 14-mL tube (marked with graduations). Add 3 mL 20 m*M* HEPES, pH 7.2, and vortex.
2. Centrifuge at 1000*g* for 2 min. Most of the beads will be sedimented, but a small fraction will rise to the top.
3. To remover the buffer, depress the plunger of a 1-mL pipetor and then place the tip into the bead suspension. Slowly raise the plunger to draw off the buffer. Repeat this until the buffer has been removed such that the beads are barely covered.
4. Add 3 mL of the same buffer, mix, and recentrifuge. Remove the buffer until the remaining volume of buffer/beads is approx 2.5 mL.

3.5.2. Preparation of Phospholipids

1. Place 50 mg asolectin in a glass scintillation vial. Add 1 mL chloroform and agitate gently to dissolve. Dry off the solvent under a stream of nitrogen under a fume hood.
2. Redissolve the lipids in 1 mL diethylether and dry as above. The lipid should now form a thin layer on the base of the vial.
3. Place a small stir bar in the vial and then add 650 μL of 20 m*M* HEPES, pH 7.2 plus 100 μL of octylPOE (*see* **Note 18**). Place in a 50°C water bath. Periodically, remove the vial from the heat and stir briskly for approx 2 min, before replacing in the water bath. After about 30 min of this heating/stirring, all the lipid will be in solution.
4. Allow the lipid/octylPOE solution to cool to room temperature.

3.5.3. Preparation of L-CPT I Proteoliposomes

1. Combine 700 μL of the lipid/octylPOE solution with 300 μL of the purified L-CPT I/Triton X-100 solution in a microfuge tube.
2. Transfer the mixture to the tube containing the BioBeadsSM2 and flick the tube to mix.
3. Rock gently for 1 h at room temperature (*see* **Note 19**).
4. Transfer the contents of the tube to a disposable plastic spin column. Transfer of the bulk of the slurry is best achieved using a disposable plastic transfer pipette, which has been truncated to widen the opening. To remove the last of the slurry, invert the tube at 45° and slowly gather the remaining material with a steel microspatula and drag it to the tip of the tube and into the spin column reservoir.
5. Place the spin column in a 14-mL tube and centrifuge at 1000*g* for 2 min. The collected effluent containing the proteoliposomes will be milky in appearance.
6. Divide the proteoliposome suspension into aliquots, freeze in liquid nitrogen and store at −70°C. Under these conditions there is no significant loss of CPT I activity even after 6 mo of storage.

3.6. Removal of Detergents and Lipids for Protein Assay

High concentrations of certain lipids and detergents can interfere with colorimetric protein determinations. To avoid this problem we have developed the following method for sample preparation and protein assay, using the BCA Protein Assay Kit from Pierce. This method is effective for measurement of protein in all the fractions obtained during the harvest of yeast cells, L-CPT I purification and reconstitution procedures described in this chapter.

1. Prepare ethanol/diethylether mixtures in solvent-resistant tubes: 3:1 (3 mL/1 mL), 1:1 (2 mL/2 mL), 1:3 (1 mL/3 mL), pure ether (4 mL). Place in flash-proof −20°C freezer.
2. Adjust protein samples to a volume of 50 mL with 20 m*M* HEPES, pH 7.2 in 1.5-mL microfuge tubes.
3. Add 250 mL of 3:1 ethanol:ether, vortex and place at −20°C for 30 min.
3. Centrifuge at 16,000*g* in a microfuge at 4°C for 10 min.
4. Discard the supernatant.
5. Repeat **steps 3–5** with ethanol:diethylether at 1:1, then 1:3 and finally with pure ether.
6. Allow the final pellet to air-dry for 5 min.
7. Resuspend with 50 μL of 5 % SDS/0.1 *N* NaOH.
8. Perform BCA protein assay as per the manufacturer's instructions.

4. Notes

1. The CPT assay described is generally applicable to CPT I or CPT II activity from any source. If the assay is to be applied to other biological material (e.g., mito-

chondrial fractions from animal tissues or homogenates of cultured cells), care should be exercised in sample preparation such that the mitochondria remain largely intact. Direct assay of such material will measure only CPT I, which is exposed on the mitochondrial surface. For assay of CPT II, the detergent octylglucoside is added to 1% (w/v) from a 20% stock. This solubilizes the mitochondrial membranes, inactivating CPT I, but releasing the detergent stable CPT II from the mitochondrial matrix. Assay of the octylglucoside containing sample, therefore, gives a measure of CPT II.

2. An alternative CPT assay has been described, in which the CoASH released from palmitoyl-CoA by the enzyme is reduced with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which can be detected as a change in absorbance at 412 nm ***(33)***. Whereas this assay has the advantage of rapidity, it performs poorly for studies where malonyl-CoA inhibition is a focus, since any lysis of the malonyl-CoA, releasing free CoASH, will cause an artifactual change in absorbance. This results in an underestimate of inhibition.
3. Although the substrate concentrations described are those we routinely employ, the assay can be performed with different concentrations of either carnitine or palmitoyl-CoA (e.g., for the determination of kinetic parameters). However, we recommend that the amount of [^{14}C]carnitine should be held constant. At low substrate concentrations, careful time courses are necessary to ensure linearity.
4. The Substrate Mix is stable if stored at −20°C and placed on ice when thawed. Assay tubes containing the Substrate Mix should not be prepared more than an hour before assay. If the Substrate Mix is allowed to remain at room temperature for extended periods, we have noticed a significant rise in the blank value.
5. Components of the Assay Cocktail are included for the following reasons: BSA is necessary to bind the palmitoyl-CoA substrate, which otherwise has detergent effects. ATP and $MgCl_2$, substrates for acyl-CoA sythetase, are included to minimize deacylation of the palmitoyl-CoA substrate. Glutathione enhances enzyme stability. KCN and rotenone inhibit the electron transport chain, and hence prevent significant further metabolism of the palmitoyl-carnitine product, when using intact mitochondria. Clearly, ATP, $MgCl_2$, KCN, and rotenone could be omitted from the reaction when assaying the final proteoliposome product.
6. The entire manual for the EasySelect *Pichia* Expression Kit is available from the Invitrogen web site. This reference manual discusses the *P. pastoris* expression system and the use of different vectors and host strains and provides detailed protocols for handling *P. pastoris* as an expression system.
7. The Qiagen Plasmid Midi Kit is suitable for purification of up to 100 μg of plasmid DNA. The product is of sufficient quality for direct sequencing or yeast transformation.
8. Baffled culture flasks are available from Kontes Glass. The presence of the baffles significantly improves aeration of the culture and enhances the growth rate.
9. We have experimented with the use of [^{3}H]carnitine in place of the [^{14}C]-labeled substrate, in an attempt to reduce costs. However, after trying preparations from several suppliers, we have found that the background (blank) value is unacceptably high.

10. We routinely use ScintiSafe30% scintillant, but most other miscible preparations should be suitable.
11. Typically, the fractions obtained in this purification protocol will require dilution before assay. For example, we routinely dilute the total cell homogenate (**Subheading 3.4.2., step 15**) or resuspended membrane pellets (**Subheading 3.4.3. step 1**) 50-fold and the final proteoliposomes 10-fold and assay over 8 min.
12. The preferred context for the initiator methionine in yeast is not the same as that in mammals ***(34)***. In the case of rat L-CPT I, we used the oligonucleotide primer to modify this region from the original CAAG*ATG* to AATA*ATG*.
13. Initiation of translation in yeast is highly sensitive to secondary structure of the mRNA in the leader region ***(34)***. The pPICZ polylinker consists of 10 restriction enzyme sites, largely palindromic and hence predisposing towards secondary structure. To avoid possible problems, we chose to insert the L-CPT I cDNA at the most 5' site in the polylinker *Sfu I*. Although not definitive evidence that this was the problem, our experience with expression of the alternative isoform, M-CPT I, was that expression was barely detectable when the cDNA was placed in the *Sac II* site (the second most 3' of the 10 unique sites in the polylinker), but that when the cDNA was inserted into the *Sfu I* site, expression levels were comparable to those we had achieved for L-CPT I.
14. Transformation of *P. pastoris*, with chromosomal integration of the expression cassette, is achieved by homologous recombination in the *AOX1* region, using linearized plasmid DNA. The pPICZ series of plasmids have three unique restriction sites designed for the purpose of plasmid linearization (*Sac I*, *Pme I*, *BstX I*).
15. In some instances (generally less than 1% of transformants), multiple copies of the expression cassette become integrated into the *P. pastoris* genome. It is possible to select for these transformants using higher concentrations of Zeocin. However, in order to obtain a sufficient number to screen in this way, it is advisable to use a more efficient method of transformation, such as electroporation.
16. As a crude estimate of relative cell number, we often describe the extent of culture growth in terms of an absorbance at 600 nm. In order to read an accurate absorbance, it is frequently necessary to dilute the culture. The suggested dilutions given should yield an absorbance after dilution of less than 1.0.
17. To minimize breakage during the spheroplasting procedure, centrifugation speeds are the lowest, which will effectively sediment the cells. The low speed, combined with DNA released from a few cells which are inevitably broken during the removal of the cell wall, results in a very loose, stringy pellet. With care, however, most of the supernatant can be poured off effectively. The DNAse I added during the lyticase digestion helps to ameliorate this problem.
18. For accurate pipeting of viscous solutions such as octylPOE, we recommend the use of the Microman positive displacement pipette from Gilson–Rainin.
19. The temperature at which this step is performed has a profound influence on the malonyl-CoA sensitivity of the reconstituted product ***(17)***. We have measured an IC_{50} (concentration of inhibitor giving 50% activity) of 11 μM for enzyme reconstituted at room temperature compared to 32 μM if this is performed at 4°C ***(17)***. The

lipid composition of the membrane is believed to affect malonyl-CoA sensitivity, and we believe this effect is due to formation of proteoliposomes of differing lipid content, resulting from the phase behavior of the individual components of the asolectin phospholipid mixture at different temperatures. The kinetics regarding to carnitine and palmitoyl-CoA are not affected by reconstitution temperature.

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24

Crystallization in Lipidic Cubic Phases

A Case Study with Bacteriorhodopsin

Valentin I. Gordeliy, Ramona Schlesinger, Rouslan Efremov, Georg Büldt, and Joachim Heberle

1. Introduction

Twenty to thirty percent of proteins coded by the genome are membrane proteins ***(1)***. They form pumps and channels in order to control and guide transport of ions and metabolites. Other membrane proteins function as receptors and are responsible for molecular recognition of hormones and neurotransmitters. In spite of strong efforts, it is extremely difficult to crystallize these proteins and only a few different integral membrane proteins have been crystallized so far ***(2)*** and modeled at high resolution (*see* http://www.mpibpfrankfurt.mpg.de/michel/public/memprotstruct.html). Even in the case of water soluble proteins, for which good approaches to crystallization have been established, considerable efforts are necessary to search for the proper crystallization conditions by screening over a wide range of different parameters (pH, ionic strength, precipitants, protein concentration, etc.). In the case of membrane proteins, one faces even greater problems. Membrane proteins are amphiphilic in nature; in order to be solubilized the use of detergents is inevitable. A major obstacle to overcome is finding the detergent that preserves the stability of the protein. Unfortunately, "what seems to be suited for crystallization of membrane proteins is less suited for their stability" ***(3)***.

A striking example of the problems one faces in this field is the history of crystallization of a small membrane protein, Bacteriorhodopsin (bR). From the first publication on three-dimensional (3-D) crystals ***(4)*** it took about 20 yr to grow crystals of bR that diffract to high resolution ***(5,6)***. The recent success in crystallization of this integral membrane protein is intimately tied to the devel-

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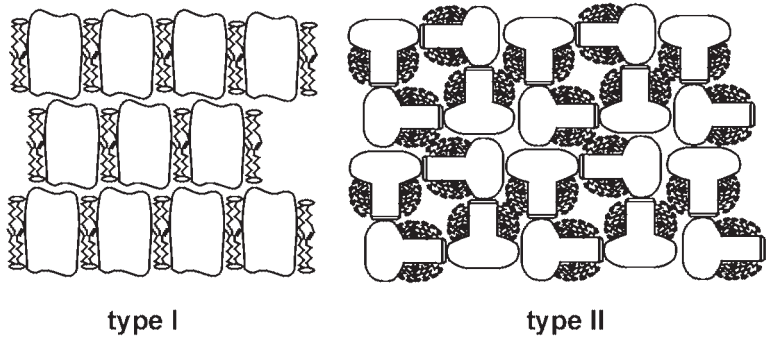

Fig. 1. Type I and type II of membrane protein crystals

opment of the novel concept of crystallization in lipidic bicontinuous cubic phases ***(7)***. Over the past 5 yr, this novel approach has been intensively exploited by several groups and has led to tremendous progress in the resolution of the bR structure, peaking now at 1.55 Å ***(8)***. Structures of the reaction intermediates have been solved as well ***(9,10)***. In addition, halorhodopsin ***(11)*** and sensory rhodopsin II ***(12)*** have been crystallized by the same approach and the structures of these proteins have been solved ***(13)***. Although the elucidation of the mechanism of crystallization in lipidic matrices still needs further exploration ***(14,15)***, the efficiency of the new method is striking. For the first time, crystals of type I (*see* **Fig. 1**) have been grown that diffracted to high resolution. This approach is especially important because conventional methods of protein crystallization often fail for the predominantly apolar membrane proteins owing to geometrical restrictions imposed by relatively large detergent belts around their hydrophobic core preventing proper inter-protein contact of their hydrophilic surfaces.

Despite the fact that the structure of membrane proteins has been solved with crystals grown in lipidic cubic phase, a detailed practical protocol of the crystallization procedure has not yet been published. Today, the crystallization of bR is well-established and the presented methodology can serve as a guide for those researchers who would like to use lipidic cubic phases to crystalize other membrane proteins.

The crystalline arrangement of the protein might induce structural alterations that influence the enzymatic mechanism. In the worst case, the physiological function of the protein is inhibited in the crystal. Therefore, an assay on the functionality is in demand ***(16)***. With the vectorial functions of membrane proteins, this is as intricate as it is significant because usually no chemically altered reaction products are formed. It is shown by microspectroscopy on a single crystal of bR that intermediate states can be trapped at low tem-

peratures. The difference spectra in the visible and the infrared wavelength range are virtually indistinguishable to those of the protein in the native environment. Thus, this methodology provides clear evidence that the structure in the 3-D-crystals is biologically relevant. To facilitate trapping of the intermediate states bR mutants are often employed. Because Cline and Doolittle ***(17)*** established a transformation procedure for halobacteria, it is advantageous to retransform modified bR genes and express them homologously. This allows purification of mutated bR with its natural lipid environment, the purple membrane.

2. Materials

2.1. Cultivation of Halobacterium salinarum

1. Pepton medium: 10 g/L pepton (1%; L37 from Oxoid), 250 g/L NaCl (4.3 *M*), 20 g/L $MgSO_4 \cdot 7H_2O$ (80 m*M*), 2 g/L KCl (27 m*M*), 3 g/L $Na_3citrate \cdot 2H_2O$ (10 m*M*), adjust to pH 6.5 with NaOH. Sterilize for 2 h at 90°C in a dry sterilizer.

2.2. Isolation of Purple Membrane

1. Basal salt solution: 250 g/L NaCl (4.3 *M*), 20 g/L $MgSO_4 \cdot 7H_2O$ (80 m*M*), 2 g/L KCl (27 m*M*).

2.3. Transformation of Halobacterium salinarum

1. Support medium.
   a. Dissolve 5 g tryptone and 3 g yeast extract in 200 mL H_2O and autoclave to sterilize.
   b. Dissolve 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 3 g $Na_3citrate \cdot 2H_2O$, 2 g KCl, 25 mL 2 *M* Tris-HCl-pH 7.4 in 897 mL H_2O and autoclave to sterilize.
   c. 500 m*M* $CaCl_2$.
   d. Mix components (a) and (b) and supplement with 3.1 mL component (c) to give a volume of 1.1 L.
2. Support medium agar plates.
   a. Mix 5 g tryptone, 3 g yeast extract and 15 g agar with 200 mL H_2O in a 1L bottle and dissolve slowly in a microwave oven.
   b. Dissolve 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 3 g $Na_3citrate \cdot 2H_2O$, 2 g KCl, 25 mL 2 *M* Tris-HCl pH 7.4 in 897 mL H_2O, and autoclave to sterilize.
   c. 500 m*M* $CaCl_2$.
   d. Pour component (b) (cooled to 60°C after autoclaving) into the bottle with component (a) and supplement with 3.1 mL component (c) Depending on the shuttle vector add Novobiocin to a concentration of 1 μg/mL or Mevinolin to a concentration of 50 μ*M*, mix well and pour into Petri dishes.
3. Spheroplast dilution solution: Dissolve 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 3 g $Na_3citrate \cdot 2H_2O$, 2 g KCl, 25 mL 2 *M* Tris-HCl pH 7.4, 3.1 mL 500 m*M* $CaCl_2$, and 150 g sucrose in 1 L H_2O and filter sterilize.

4. Spheroplast buffer (SPH): Dissolve 11.6 g NaCl (2 *M*), 0.2 g KCl (25 m*M*), 5 mL 1 *M* Tris-HCl pH 8.75 (50 m*M*) and 15 g sucrose in 100 mL H_2O and filter sterilize.
5. Ethylanediaminetetraacetic acid (EDTA) solution: 500 m*M* EDTA in SPH, pH 8.75.
6. PEG solution: 6 mL PEG 600 in 4 mL SPH.
7. Regeneration medium: Supplement support medium with 15% sucrose and filter sterilize.

2.4. Solubilization and Crystallization of Bacteriorhodopsin

1. Solubilization buffer: 20 m*M* Na_2HPO_4/KH_2PO_4 (Na/K-P_i), pH 6.9, 3.75 % (w/v) octyl-β-D-glucoside (OG, *see* **Note 1**).
2. Microconcentrator (centricon YM-30, Millipore, USA).
3. Crystallization buffer: 368 m*M* Na/K-P_i pH 5.6, 1.2% (w/v) OG.
4. Monoolein (1-Monooleoyl-rac-glycerol, Sigma Chemicals).
5. 200 μL tubes (Biozym).
6. Sample box thermostatted at 22°C.

2.5. Microspectroscopy

A commercial infrared microscope (A590, Bruker, Germany) was modified to record absorption spectra in both the visible and the infrared range for the same sample (*see* **Fig. 2**). The light source is either the halogen bulb of the microscope's observation light (for measurements in the UV/Vis) or the globar from the FT-IR spectrometer (Bruker Vector 22, for infrared (IR) spectroscopy). The respective radiation source was selected by a movable mirror (MM1). The concave mirror (CM) focuses the light on the crystal sample. An aperture wheel with different aperture sizes limits the observation plane to diameters between 20 μm and 400 μm. For experiments in the visible wavelength range, a movable mirror is set into the light path to reflect the light beam into the binocular. One ocular is used for visual inspection of the sample to adjust the sample within the light path. The other ocular is interfaced via an optical fiber to a spectrograph with an intensified charge coupled device (CCD) camera as detector (Scientific Instruments, Germany). For the recording of IR spectra, MM2 is moved out of the light path and the transmitted intensity is measured by a liquid nitrogen (LN_2) cooled mercury cadmium telluride (MCT) detector. The entire microscopic setup allows the measurement across the range of 380–14.300 nm (corresponding to 26.316–700 cm^{-1}) of samples as small in diameter as 20 μm. IR experiments of smaller particles are diffraction limited. The crystal was mounted in a cryoloop (Hampton Research). The temperature of the sample was controlled by a nitrogen-cooled cryostream system (Oxford, UK) perpendicular to the measurement beam (not shown).

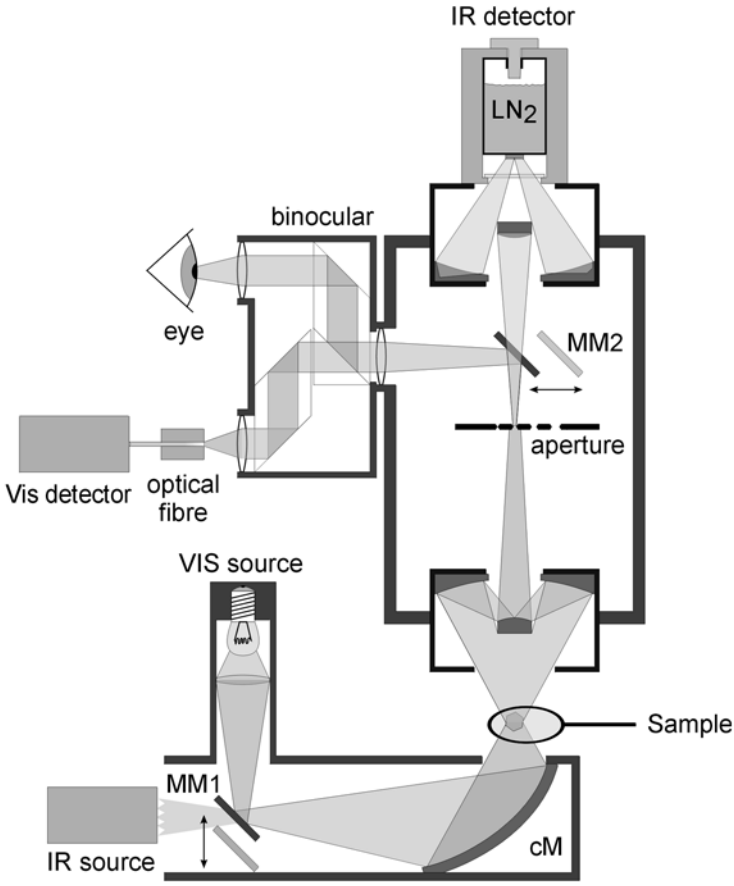

Fig. 2. Optical scheme of the microspectrometer used for the characterization of the crystals of bacteriorhodopsin. The use of focusing mirrors instead of lenses (Cassegrain optics) allows to use the whole wavelength range from mid-infrared to ultraviolet. The modulated emission from an FT-IR spectrometer (Vector 22, Bruker, Germany) is used as the IR source. Alternatively, emission from a halogen bulk that is usually used for sample observation can be employed by insertion of a mirror (MM1, motor driven) into the light path.

3. Methods

3.1. Cultivation of Halobacterium salinarum *and Expression of Bacteriorhodopsin in Liquid Media (18)*

For the efficient expression of bacteriorhodopsin in *Halobacterium salinarum*, the strategy is to produce a large biomass before setting optimal culture conditions to induce the expression of bacteriorhodopsin. *Halobacterium salinarum* as

a chemoorganotroph is grown at best under aerobic conditions in complete media. As oxygen solubility in liquid media decreases with higher temperatures and salt concentrations, the cultures in Erlenmeyer flasks had to be shaken vigorously with low volume to allow good aeration. Be aware that extended periods of vigorous shaking leads to cell lysis. In addition, increased evaporation of water from the medium occurs and NaCl precipitates when the saturation level is exceeded.

1. Dilute a fresh starter culture in the latelog phase 1:50 in peptone medium and cultivate at optimal temperature of 37–40°C. Depending of the size of flasks shake the cultures with 120–150 rpm /min in rotary shakers with motion of Ø 50 mm (*see* **Note 2**).
2. To promote increased expression of bacteriorhodopsin, the oxygen concentration has to be reduced in the culture when reaching midlog phase after approx 2–4 d of vigorous shaking. Change the shaking motion to 80 rpm to decrease aeration.
3. Harvest the cells by centrifugation after 4–6 d.

3.2. Isolation of Purple Membrane (PM)

3.2.1. Lysis of Cells

1. Wash the pelleted cells once in basal salt solution in a volume equal to one-fifth the culture volume. To do so, resuspend the cells cautiously and harvest again by centrifugation.
2. To lyse the cells, resuspend 1 g of wet cell pellet in 25 mL 0.02% NaN_3 in H_2O, add DNAse I (approx 5 mg/L suspension) to reduce viscosity due to genomic DNA, and agitate with a magnetic stir bar overnight at 4°C.

3.2.2. Fractionating Centrifugation

To separate PM from cell debris and other impurities, a series of centrifugation steps must be carried out.

1. Fill the cell extract into centrifugation tubes and centrifuge at 4300*g* for 10 min at 4°C.
2. Transmit the supernatant into a fresh tube and centrifuge at 7600*g* for 10 min at 4°C.
3. Repeat **step 2**.
4. Transmit the supernatant into a fresh tube and centrifuge at 55,000*g* for 60 min at 4°C.
5. Remove the supernatant, resuspend the pellet in H_2O and centrifuge again as described in **step 4**.
6. Remove the supernatant, resuspend the pellet in H_2O and centrifuge at 60,000*g* for 60 min at 4°C.
7. Repeat **step 6**.
8. Resuspend the pellet in 0.02% NaN_3 in H_2O (approx 1 mL 1 g cells).

3.3. Transformation of Halobacterium salinarum

3.3.1. Cultivation of Cells

1. Inoculate 50 mL support medium with *H. salinarum* from a glycerol stock, from stationary cultures in liquid media stored in the cold room at 4°C or from agar plates.
2. Let the cells grow with vigorous shaking (approx 200 rpm) at 40°C to an OD_{600} of 0.6–0.8 (*see* **Note 3**)
3. Harvest the cells by centrifugation.
4. Resuspend the cells with caution in 5 mL SPH.
5. Add 250 μL EDTA solution to the cells and observe spheroplast formation under a light microscope.
6. Mix 200 μL of spheroplast suspension with 1–5 μg plasmid DNA in 2 *M* NaCl in a 15-mL polypropylene tube and incubate at room temperature for 20 min.
7. To the spheroplast/DNA mixture pipet the same volume of PEG solution, mix with caution (*see* **Note 4**) and let stand at room temperature for 20 min.
8. Dilute the cells with 10 mL spheroplast dilution solution and incubate for 30 min at 40°C.
9. Harvest the cells and resuspend them in 1 mL regeneration medium.
10. Shake the cells in the 15-mL tube at 37°C in a transverse way.
11. After 2 d, the cells can be put on agar plates with Novobiocin or Mevinolin, respectively. Transformants are observed after 1–2 wk at 40°C.

3.4. Solubilization of PMs (19)

1. Dilute the PM suspension with solubilization buffer to a final bR concentration of 2.5 mg/mL. This corresponds to a 15× excess of detergent over protein.
2. Stir for 2 d at 4°C in the dark.
3. To remove insoluble purple membranes, spin the sample at 90,000*g* in a centrifuge for 1 h at 4°C.
4. Collect the supernatant and concentrate the protein by the use of a microconcentrator. Use the swing-out rotor and spin at 5000*g* at 4°C for 3 h. To prevent the filter from obstruction interrupt the centrifugation in intervals of 30 min and gently mix the protein solution with a pipet.
5. Add 2.125 parts of crystallization buffer to the bR solution to replace the solubilization buffer. This results in a final phosphate concentration of 250 m*M*.
6. Concentrate the protein solution to 10 mg/mL as described in **step 4** (*see* **Note 5**). The bR concentration is determined by UV/Vis spectroscopy. The absorbance spectrum (*see* **Fig. 3**) exhibits two distinct bands at 560 nm (retinal absorption) and at 280 nm (aromatic amino acids). The absorbance at 560 nm (A_{560}) is used to calculate the concentration (in mg/mL) from the Lambert-Beer law: $c_{bR} = MW_{bR} * A_{560}/(\epsilon_{bR} * d)$ with the molecular weight MW_{bR} = 26,500, the extinction coefficient ϵ_{bR} = 42,000 $M^{-1}cm^{-1}$ for bR dissolved in OG solution ***(19)*** and d (in cm) as the optical pathlength. The purity of bR is characterized by the ratio of the absorption at 280 and 560 nm. For crystallization purpose A_{280}/A_{560} should not exceed 1.8.

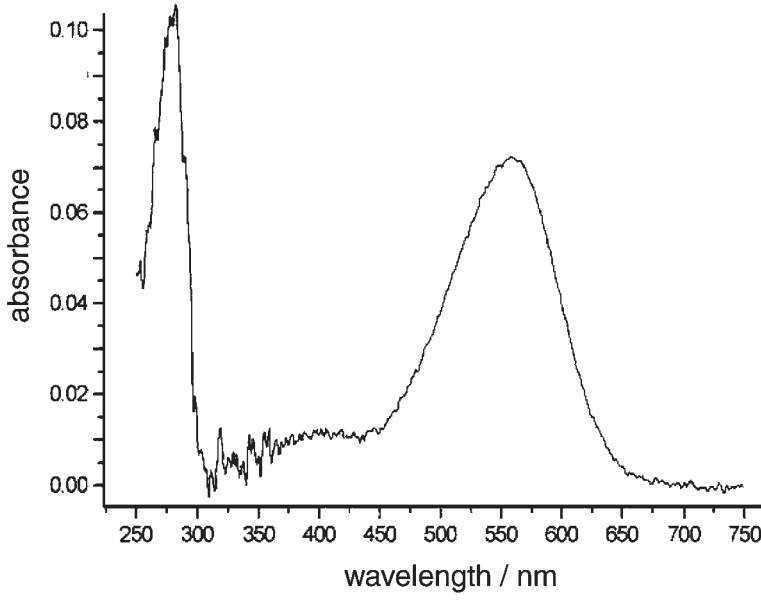

Fig. 3. UV/Vis absorption spectrum of bacteriorhodopsin solubilized in octylglucoside.

3.5. Crystallization of bR in Lipidic Cubic Phase

1. Fill the 200 μL tube with approx 4–6 mg of monoolein (MO) powder.
2. Melt MO at 40°C and spin the lysolipid down for 10 min at 13,000*g* at room temperature.
3. To obtain the isotropic lipidic phase, MO is kept for an additional 20 min at 40°C. Subsequently, let the lipidic phase cool to room temperature. The MO phase must remain transparent. If not, the melting procedure should be repeated (*see* **Note 6**).
4. Add 1 μL bR solution (10 mg/mL bR) per mg of MO (*see* **Note 7**).
5. A homogenous lipid/protein mixture as well as the formation of the cubic phase is achieved by the following centrifugation procedure. Spin the PCR tube with the sample at 22°C at 10,000 rpm for 15 min. Rotate the tube within the rotor by 90° and spin again. Repeat this spinning procedure four times to obtain a homogenous mixture.
6. Leave the sample for 24 h in the dark at 22°C.
7. Add a grinded powder of KH_2PO_4 mixed with Na_2HPO_4 (95/5 w/w) to obtain a final concentration of 1–2.5 *M* phosphate (pH 5.6).
8. Homogenize the sample by repetitive centrifugation as described in **step 4**.
9. Leave the crystallization batch in the dark at 22°C. Microcrystals of bR (10–20 mm in diameter) usually appear within 1 wk after phosphate has been added (*see* **Note 8** and **Fig. 4**).

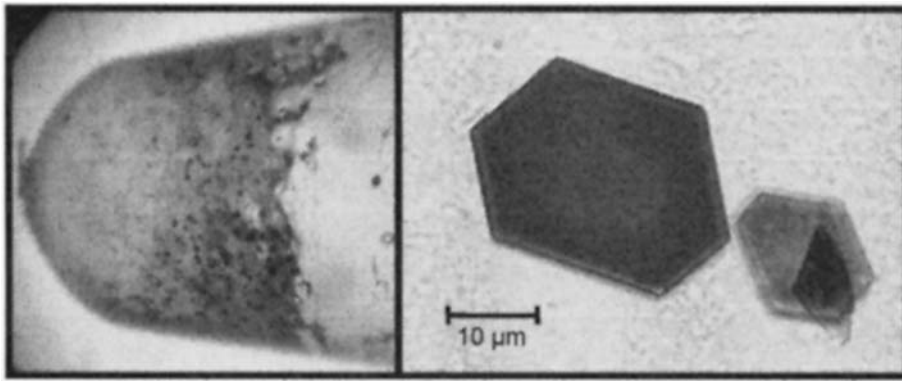

Fig. 4. Photographs of a crystallization batch of bR in the lipidic cubic phase (left panel). bR crystals are visible as purple spots. The hexagonal morphology is seen at higher magnification (right panel) of crystals that have been taken out of the crystallization tube.

3.6. Spectroscopic Assessment of the Functionality of Crystallized Bacteriorhodopsin

1. Mount a crystal of bacteriorhodopsin with a cryoloop in the microspectrometer. Preferable are those crystals that are thinner than 10 µm (*see* **Note 9**).
2. Select an aperture to match the diameter with the size of the crystal.
3. Flash-freeze the crystal by removing the block of the cryostream. The temperature of the cryostream selects the intermediate (here 240 K).
4. Record an intensity spectrum of the dark state as reference. The visible spectrum is selected by insertion of the two movable mirrors (MM1 and MM2) into the light path. The infrared spectrum is recorded by moving out the mirrors.
5. Illuminate the crystal with yellow light to trap the *M* intermediate.
6. Record the intensity spectrum in the visible as well as in the infrared range.
7. Calculate the difference absorption spectra by dividing the intensity spectrum of the dark state through the spectrum of the illuminated state and taking the decadic logarithm.
8. Compare the results with the spectra depicted in **Fig. 5**. The visible difference spectrum (upper trace) shows an absorbance decrease around 570 nm which reflects the depletion of the dark state of bR. The formed *M* intermediate exhibits an absorbance maximum at 412 nm. The corresponding infrared difference spectrum (bottom trace) shows strong negative peaks of the chromophore retinal at 1526, 1253, 1200, and 1167 cm^{-1}. The small positive band at 1761 cm^{-1} is evidence for proton transfer from the Schiff base to D85 ***(20)***.

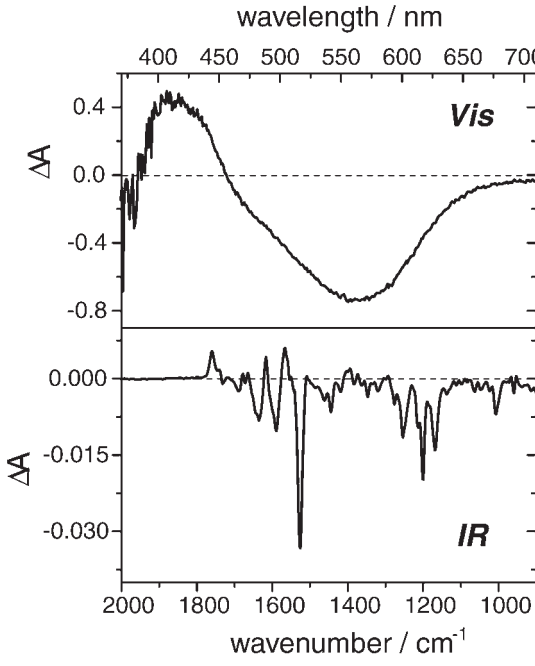

Fig. 5. Difference spectra in the visible (top) and in the mid-IR range (bottom) of a single microcrystal of bacteriorhodopsin. Spectra have been recorded after yellow light illumination at 240 K with the dark state as reference.

4. Notes

1. Octylglucoside should be stored at −20°C in dry form. In soluble form, it decomposes within several hours at room temperature and under light illumination. Therefore, all OG solutions should be stored at 4°C in the dark. It is recommended to always use a freshly prepared OG solution.
2. When already exposed to light the cells begin to produce bacteriorhodopsin visible by turning the culture color slightly red.
3. When the culture is growing too slow it is advisable to passage the cells several times, until they speed up the time of cell division. Fast growing cells seem to give better results in transformation.
4. Too careless mixing leads to lysis of some cells, seen in the light microscope as long filaments caused by released genomic DNA and subsequent occupying by spheroplast. This phenomenon reduces transformation efficiencies.
5. Solubilized protein should be stored at −80°C. Handling of bR solution should be done at 4°C. Avoid extended light exposure!
6. It is mandatory to prevent the formation of the turbid crystalline lipid phase. Otherwise, the following reconstitution of bR into the lipidic phase will result in an

inhomogeneous distribution. Yet, an homogenous cubic phase is crucial for the growth of high-quality crystals.

7. bR solution should be warmed to 22°C prior to adding to the lipid. This is important for the preservation of the cubic phase (*see* **ref.** ***14*** for the phase diagram of MO) and for the uniform distribution of the protein within.
8. The PCR tubes used for crystalization should be transparent to facilitate sample inspection under the light microscope.
9. The presence of the lipidic cubic phase can lead to distortions in the spectroscopic experiments. The lipidic phase is removed by dipping the crystals in octylglucoside solution. Alternatively, monoolein can be cleaved by lipase ***(21)***.

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25

Optical Biosensor Assay Using Retroviral Receptor Pseudotypes

Joseph Rucker

1. Introduction

Optical biosensors are a class of analytical instruments that can provide real-time quantitative information on the interaction of proteins or other biological macromolecules. One of the most commonly used optical biosensor technologies is based on surface plasmon resonance (SPR), which measures changes in refractive index at surfaces ***(1)***. A change in refractive index at the surface of an SPR-biosensor is proportional to an increase or decrease in mass at its surface. Protein–protein interactions are measured by tethering one protein to the biosensor surface while monitoring changes in refractive index upon exposure to a soluble protein, reflecting either binding, which corresponds to an increase in refractive index, or dissociation, which corresponds to a decrease in refractive index. Biosensor experiments can be used to quantify binding equilibrium constants (K_D), as well as association and dissociation rate constants (k_{on} and k_{off}). They can be significantly more sensitive than classical radioligand binding assays and can detect interactions with K_D in the range of 10^{-4}–10^{-12} *M*.

An SPR-based biosensor surface is composed of a thin gold film on a glass surface. The gold surface is usually functionalized with a self-assembled thiol monolayer ***(2)***. Commonly utilized surface includes carboxylated dextran and straight-chain alkanethiols with terminating carboxyl groups ***(3)***. Proteins and other biomolecules can be tethered to carboxylated surfaces using a variety of coupling techniques. For example, carboxyl groups on the biosensor surface can be converted to an activated NHS (N-hydroxysuccinimide) ester using EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide). Proteins are then covalently

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coupled to the activated NHS-ester via lysine residue side chains or amino-termini. Numerous other coupling technologies also exist ***(2)***.

Whereas optical biosensors have been shown to be a powerful technique for understanding protein–protein interactions of soluble proteins, integral membrane proteins have been more difficult to study. Although GPI-linked and single-pass transmembrane proteins can be expressed as soluble ectodomains ***(4,5)***, multispanning proteins such as G protein-coupled receptors (GPCRs) have proven to be more intractable (*see* **ref. *6*** for an example). In addition, binding phenomena that are dependent on the membrane context, such as ligand-induced receptor dimerization, are difficult to study using traditional biosensor methods ***(7)***. New methods are obviously needed for membrane receptor presentation in the context of biosensor assays.

One novel method recently developed for membrane receptor presentation is the use of retroviral pseudotypes. It is well known that for many lipid-enveloped viruses (including retroviruses), the viral envelope glycoprotein (Env) responsible for mediating membrane fusion and viral entry can be replaced with the Env protein from a heterologous virus ***(8)***. This can be accomplished by either replacing the Env protein in the viral genome or by supplying the heterologous Env protein *in trans* to an Env-deficient virus, generating what is known as a pseudotyped virus. Env can also be replaced by non-Env proteins expressed on the cell surface, generating what are referred to as receptor pseudotypes ***(9,10)***. A large number of receptors with diverse membrane topologies has been incorporated into pseudotypes, including proteins with single and multiple transmembrane domains.

A recent study has demonstrated that retrovirus-derived receptor pseudotypes can be used as receptor display vehicles for surface plasmon resonance (SPR)-based biosensor studies of receptor-ligand interactions ***(11)***. In these particular experiments, pseudotypes composed of the Gag protein of murine leukemia virus (MLV) and containing the G protein-coupled receptors (GPCRs) CXCR4 and CCR5 were covalently tethered to a biosensor surface (*see* **Fig. 1**) and were shown to be competent to bind a variety of ligands. Pseudotype-based biosensor assays can thus provide a method for studying membrane receptor-ligand interactions.

The purpose of this chapter is to provide methods for preparing and characterizing receptor pseudotypes and for attaching pseudotypes to biosensor surfaces. Details are specific for Biacore™ biosensors, which are currently the most widely used SPR-based instrument. It should be noted that other methods for studying membrane-bound proteins using biosensor technology have also been explored ***(6,12,13)***. Similarly, receptor pseudotypes could potentially be used in nonbiosensor applications such as fluorescence anisotropy.

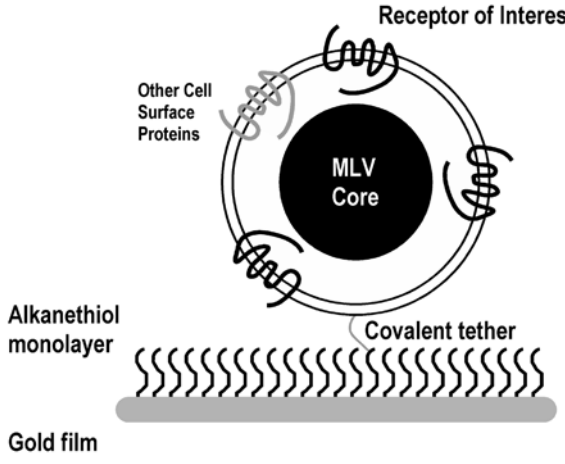

Fig 1. Schematic of a purified receptor pseudotype attached to a biosensor surface. A variety of alkanethiol monolayers and methods of tethering exist.

2. Materials

2.1. Cell Culture and DNA Transfection

1. Complete Dulbecco's Modified Eagle's Medium (DMEM): DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, and 2 m*M* penicillin/streptomycin.
2. Plasmid DNA encoding for MLV *gag-pol*: pHit60 or pCGP (*see* **Note 1**).
3. Plasmid DNA encoding for receptor of interest.
4. 10× NTE: 8.77 g NaCl, 10 mL 1 *M* Tris-HCl (pH 7.4), 2 mL 0.5 *M* ethylenediamine tetraacetic acid (EDTA), water to 100 mL.
5. 0.5 *M* HEPES (pH 7.10 ± .03) (*see* **Note 2**).
6. 2 *M* NaCl.
7. 1 *M* Na_2HPO_4 (pH 7.0).
8. 2 *M* $CaCl_2$.
9. Tissue culture (TC) water.
10. Transfection buffer: 1 mL 0.5 *M* HEPES (pH 7.1), 8.1 mL TC water, 0.9 mL 2 *M* NaCl, and 20 μL 1 *M* Na_2HPO_4 (pH 7.0). This buffer is stable for several weeks or longer at room temperature.
11. 1 *M* sodium butyrate (*n*-butyric acid, sodium salt) stock solution (store at 4°C) (*see* **Note 3**).
12. 225-cm^2 plastic TC flasks.
13. 37°C, 5% CO_2 humidified incubator.

2.2. Purification and Characterization of Receptor Pseudotypes

1. Phosphate-buffered saline (PBS) pH 7.4 (*see* **Note 4**).
2. 10 m*M* HEPES, pH 7.0 (0.2 μm filtered).
3. 20% sucrose in PBS (0.2 μm filtered).
4. Beckman ultracentrifuge or equivalent.
5. SW28 and SW40 rotors or equivalent.
6. 25 × 89-mm thin-wall Ultra-Clear™ centrifuge tubes (38.5 mL) (Beckman).
7. 14 × 89-mm thin-wall Ultra-Clear™ centrifuge tubes (14 mL) (Beckman).
8. 0.45-μm pore syringe filters.
9. System for running SDS-PAGE and Western blotting such as the Mini-Protean II Cell system (Bio-Rad).
10. PVDF Membrane (for example, Immobilon-P™ from Millipore).
11. Anti-receptor antibodies (*see* **Note 5**).
12. Anti-MLV Gag antibodies (*see* **Note 6**).

2.3. Attachment of Receptor Pseudotypes to Biosensor Surface

1. Biacore optical biosensor (Biacore, Uppsala, Sweden) (*see* **Note 7**).
2. Biacore C1 or F1 chip (*see* **Note 8**).
3. PBS (0.2-μm filtered and degassed) (*see* **Note 9**).
4. 0.1 *M* glycine, pH 12.0.
5. 1 *M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).
6. 0.25 *M* N-hydroxysuccinimide (NHS).
7. 0.1 *M* sodium acetate, pH 5.5.
8. 1 *M* ethanolamine, pH 8.5.

2.4. Binding Experiments

1. PBS (0.2-μm filtered).
2. DMEM, without phenol red, supplemented with 0.1% w/v Pluronic F127 (Sigma) and 0.02% w/v sodium azide (optional) (0.2-μm filtered) (*see* **Note 10**).
3. pH 5.0 regeneration solution: 0.15 *M* oxalic acid, 0.15 *M* phosphoric acid, 0.15 *M* formic acid, and 0.15 *M* malonic acid. Adjust pH to 5.0.
4. pH 9.0 regeneration solution: 0.2 *M* ethanolamine, 0.2 *M* sodium phosphate, 0.2 *M* glycine, and 0.2 *M* piperazine. Adjust pH to 9.0.
5. Chaotropic regeneration solution: 0.46 *M* KSCN, 1.83 *M* $MgCl_2$, 0.92 *M* urea, and 1.83 *M* guanidine·HCl.
6. Receptor ligand(s).

3. Methods

3.1. Cell Culture and DNA Transfection

Receptor pseudotypes are produced in cells by the coexpression of a receptor with MLV Gag protein ***(10)***. Expression of Gag alone leads to the formation of particles that will bud through the cell's plasma membrane; a viral genome is not needed. Several expression plasmids for Gag are available, both expressing

gag using a cytomegalovirus (CMV) promoter. Receptor expression plasmids should have strong promoters that will give high levels of expression. Pseudotypes are made by transient transfection of DNA into cells using either calcium phosphate or lipid-based transfection reagents. Human epithelial kidney cells (HEK293T) are used in this protocol, but any easily transfectable mammalian or avian cell line should be adequate. However, the producer cell line should not express the receptor of interest at any significant level because the production of pseudotypes not containing the receptor of interest is an important negative control in binding assays (*see* **Note 11**).

1. One day prior to transfection, HEK293T cells should be split into 225-cm^2 TC flasks such that they will be approx 50% confluent on the day of transfection. Cells should be cultured in about 30 mL of complete DMEM.
2. Transfection of a 225-cm^2 monolayer of HEK293T cells requires about 30 μg of MLV *gag-pol* expression plasmid and about 90 μg of a plasmid expressing the receptor of choice (*see* **Note 12)**.
3. A DNA cocktail is then prepared in a total volume equal to 1/20 of the volume of media covering the cells to be transfected, i.e., 30 mL of media would require 1.5 mL of DNA cocktail. The DNA cocktail is composed of NTE to 1 × (10-fold dilution), calcium to 0.25 *M* (eight-fold dilution), DNA, and water to volume.
4. DNA cocktail is added dropwise to an equal volume of transfection buffer with constant vortexing at low to medium speed in a small polypropylene tube. After a 30-min incubation at room temperature the mixture is then added to the 225-cm^2 TC flask with gentle swirling (*see* **Note 13**).
5. 4 h posttransfection, media should be replaced with fresh media. 10 m*M* sodium butyrate can also be added to increase protein expression (optional; *see* **Note 3**).
6. 48 h posttransfection, media supernatant containing retroviral pseudotypes is collected into conical tubes and cell debris removed by low-speed centrifugation and filtration through a 0.45-μm filter. Because retroviruses are approx 100 nm in diameter, use of smaller filters may lead to significant sample loss. At this point, samples can be stored for several days at 4°C.

3.2. Purification and Characterization of Receptor Pseudotypes

1. Clarified supernatant, approx 30 mL, is layered over 20% sucrose in PBS and pelleted for 90 min in an SW28 rotor at 28,000 rpm.
2. Supernatant is carefully removed to avoid contamination with serum protein and the pellet resuspended in 1 mL PBS overnight (*see* **Note 14**). The pseudotypes are then repelleted through 20% sucrose in PBS in an SW40 rotor for 45 min at 40,000 rpm. Typically, pseudotypes are diluted in 9 mL PBS total and layered over approx 4 mL of the sucrose solution.
3. Supernatant is carefully removed, and the pellet resuspended overnight in 100 uL of 10 m*M* HEPES, pH 7.0. Pseudotypes are either stored at 4°C or aliquoted and frozen at −20°C.

4. MLV-pseudotypes are normally analyzed by SDS-PAGE followed by Western blotting. Approx 5–10 μL of pseudotypes lysed in Lemmli sample buffer should give a robust signal in Western blotting.
5. Pseudotypes can also be analyzed by equilibrium density gradient ultracentrifugation using a 15%–45% sucrose gradient in an SW40 rotor at 35,000 rpm for 16 h. MLV-pseudotypes have a buoyant density of approximately 1.16 g/mL ***(10,11)***. Griffith ***(14)*** is a good source of details on ultracentrifugation.

3.3. Attachment of Receptor Pseudotypes to Biosensor Surface

1. Pseudotypes have been successfully attached to a Biacore C1 chip using both Biacore BiaX and Bia2000 biosensors. Attachments are performed using PBS (0.2-μm filtered), preferably degassed. A C1 chip is docked to a Biacore biosensor, and the flow cells were cleaned using 2 × 1.5 min injections of 0.1 *M* glycine, pH 12.0 at a flow rate of 50 μL/min followed by Biacore command EXTRACLEAN.
2. Choosing a specific flow cell, the flow rate is reduced to 5 μL min. Surface carboxyl groups are then activated by a 1:1 mixture of EDC and NHS for about 8–10 min (using Biacore command QUICKINJECT).
3. At this point, the flow rate is lowered to 2 μL/min. Using manual injection, the pseudotypes (mixed 1:1 with 0.1 *M* sodium acetate, pH 5.5) is injected over the flow cell until the desired attached RU values are reached (2000 RU to 6000 RU). This should take between 5–10 min and requires about 20 μL of the pseudotype mixture.
4. The flow rate is then reset to 5 μL/min and 35 μL of 1 *M* ethanolamine, pH 8.5 is injected over the surface of the chip to block any remaining activated carboxyl groups.
5. This process should be repeated for each surface derivatized. It is critical that a negative-control surface containing pseudotypes not expressing the receptor(s) of interest be included. Care should be taken to have approximately equivalent amounts (response units) of pseudotypes attached to each flow cell.
6. After all surfaces are derivatized, the machine is then washed extensively with PBS (using the PRIME function) until the baseline has stabilized.

3.4. Binding Experiments

A complete discussion of Biacore-based binding experiments is beyond the scope of this review (see review by Morton and Myszka ***(15)*** for a more complete discussion). However, a few general comments are in order. Proper ligand-binding studies require the measurement of equilibrium binding and association/dissociation rates at a variety of ligand concentrations and conditions. Because biosensors measure all changes in refractive index at the biosensor surface, special care must be taken so that only specific interactions between ligand and its cognate receptor are measured. Reliable binding experiments should minimize nonspecific binding of ligands, as determined by the ability of a ligand to bind to pseudotypes lacking the receptor of interest. Because pseudotypes containing the receptor of interest can be run simultaneously with negative-control pseudo-

types, low levels of nonspecific binding can be easily handled by mathematical subtraction of the negative-control signal from the pseudotype of interest. The choice of running buffer can also influence levels of nonspecific binding. Some ligands show little or no nonspecific binding with PBS, whereas other proteins require the use of running buffer additives that reduce nonspecific binding (*see* **Note 10**).

After a cycle of ligand association/dissociation is completed, bound ligand needs to be stripped from the pseudotype surface. The ability to regenerate surfaces allows for ease of varying ligand concentration and/or binding conditions. Subheading 2.4. lists a variety of regeneration solutions that can be used either singly or in combination to remove bound ligand and return the pseudotype-biosensor surface to its original (and structurally intact) state. Regeneration buffers should not contain detergents that will disrupt pseudotypes. In addition, extremes of pH appear to disrupt receptor/pseudotype integrity, although pH limits have not been rigorously defined. The choice of running buffer and regeneration conditions are totally dependent on the particular ligand-receptor pair being studied and will need to be optimized for each system studied.

Fig. 2A shows sample data for a CXCR4-specific antibody binding to the GPCR CXCR4 followed by regeneration of the pseudotype surface (details of this experiment are given in **ref.** ***11***). The signal change seen in the negative control surface is solely a result of differences in refractive index between running buffer and the antibody solution, which can easily be minimized by exchange of the antibody solution into running buffer. Aside from this bulk refractive index change, this system shows no nonspecific binding. **Fig. 2B** details the specific binding curve obtained by subtracting binding to the negative control surface.

1. The biosensor surface is equilibrated in the running buffer of choice, first by using the Biacore command PRIME (to flush out all parts of the microfluidics system) followed by flowing running buffer over the surface at 5 μL/min until the baseline signal stabilizes.
2. Flow rate is set to the highest level, given sample availability, to avoid artifacts owing to mass transport (*see* **Note 15**). Flow rates around 30 μL/min are commonly used.
3. Ligand binding is then measured by injecting a volume of sample sufficient to allow for a 1–2 min association phase, although longer association phases can also be measured in those cases where the association rate is very slow or where equilibrium binding is being measured.
4. After association, ligand is allowed to dissociate for 1–2 min, although longer periods can be used in cases of ligands with extremely slow dissociation rates.
5. After sufficient dissociation data has been collected, flow cell(s) are regenerated back to baseline values using several short injections of regeneration buffer at high

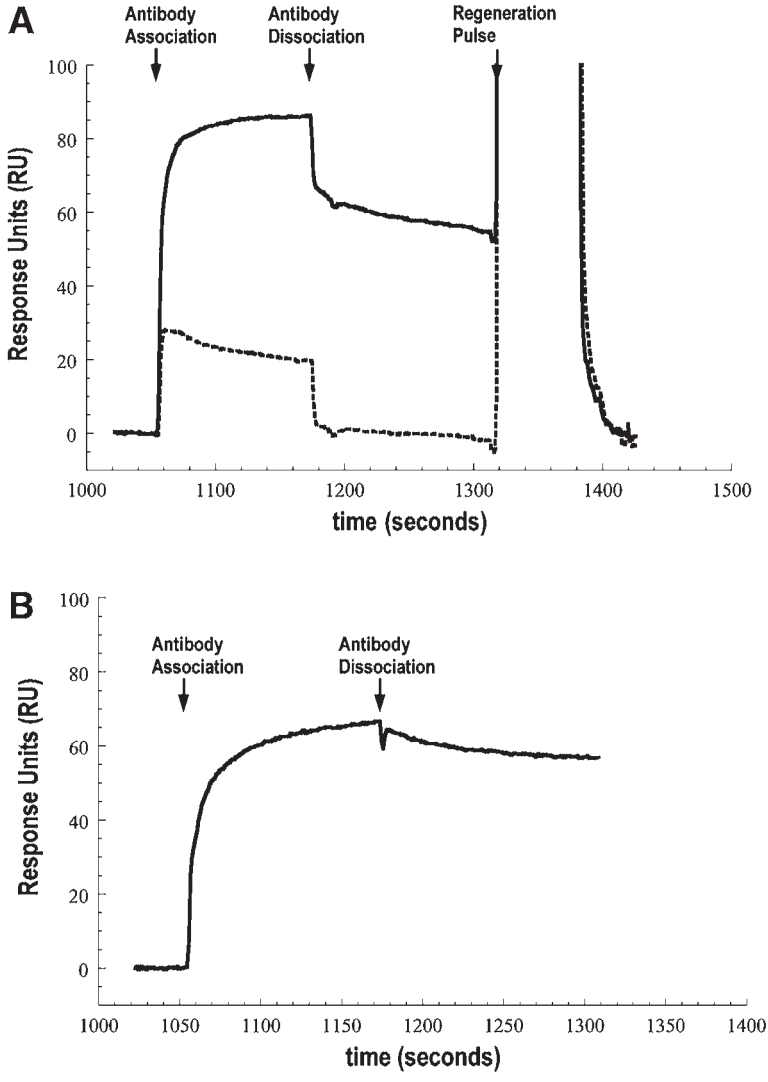

Fig. 2. Binding of an anti-CXCR4 antibody to MLV-pseudotypes expressing the chemokine receptors CXCR4 and CCR5 (*see* **ref.** ***11*** for details). **(A)** Antibody was simultaneously flowed over two flow cells containing CXCR4-pseudotypes (solid) and CCR5-pseudotypes (dashed), respectively. The signal change seen in the negative control surface is solely caused by differences in refractive index between running buffer and the antibody solution. **(B)** Subtraction of the CCR5 signal from the CXCR4 signal gives specific association and dissociation curves. Collection of binding and association data at various ligand concentration scan be mathematically analyzed to yield association and dissociation rates as well equilibrium binding data.

flow rate. For example, antibodies bound to pseudotypes expressing the chemokine receptors CCR5 or CXCR4 were effectively removed by duplicate 20 μL pulses at 100 μL/min of a 1:1 mixture of a pH 5.0 buffer and chaotropic agents.

6. Steps 3–5 are repeated at different ligand concentrations. Optimally, ligand concentrations should cover a wide range bracketing the K_d of the ligand. It may take several initial rounds of binding and regeneration for the system to come to complete equilibrium and to give highly reproducible results.
7. Once data is collected and processed (subtraction of background signal and removal of injection artifacts), equilibrium and rate constants can be analyzed by several available software packages (*see* **Note 16**). Equilibrium and kinetic data are often analyzed globally (all data sets simultaneously). Data analysis requires a choice of a particular binding model. A common default is the assumption of 1:1 binding, meaning that one ligand molecule binds to one receptor molecule. However, more complicated but sometimes more accurate models can be used, taking into account mass-transport, bivalent binding, conformational changes, and so on ***(16)***.

4. Notes

1. Both pHit60 ***(17)*** and pCGP ***(18)*** produce high levels of MLV-Gag and thus of pseudotypes. However, pCGP is somewhat more stable in bacteria and appears to produce slightly higher levels of MLV-Gag.
2. The pH of the HEPES buffer is absolutely critical for effective transfections. Small deviations from 7.10 can lead to significant reductions in transfection efficiency.
3. Sodium butyrate upregulates transcription of CMV (and other) promoters ***(19)***. However, the corresponding increase in protein production can be somewhat offset by a decrease in cell viability due to butyrate-induced cell death.
4. Unpublished studies suggest that PBS can destabilize pseudotypes under certain conditions. To avoid this possibility, HEPES-buffered saline (HBS) can be used in lieu of PBS.
5. Not all antibodies are suitable for Western blotting due to receptor denaturation.
6. Polyclonal serum against MLV-Gag was originally obtained from a recently closed NCI repository. However, hybridomas expressing anti-MLV-Gag monoclonal antibodies are available from the American Type Culture Collection. In addition, MLV-Gag present in receptor pseudotypes can be visualized by silver staining of SDS-PAGE gels.
7. Biacore BiaX and Bia2000 optical biosensors have been used for receptor pseudotype experiments. However, there is nothing precluding use of this technology with other commercial biosensors.
8. A Biacore C1 chip is a gold surface derivatized with a carboxylated alkanethiol whereas the F1 chip is derivatized with a short carboxydextran matrix. The normal carboxydextran chip (CM5) which is a *de facto* standard surface for biosensor experiments shows very poor pseudotype attachment, presumably caused by either by poor penetration of particles into the dextran matrix or extensive charge repulsion of negatively charged pseudotypes from the highly carboxylated dextran matrix. Other surface chemistries have not been extensively explored. A surface

that has potential use is the Biacore L1 chip, which contains hydrophobic residues and has been shown to bind membranes.

9. Many biosensor applications use PBS containing 0.005% Tween-20 added to reduce nonspecific binding. However, this amount of Tween-20 is sufficient to disrupt pseudotypes and should be avoided, as should the use of any potentially lytic additive.
10. In **ref.** ***(11)***, DMEM supplemented with 0.1% w/v Pluronics F-127 was able to reduce nonspecific binding in certain applications. Pluronics have been extensively studied for their ability to reduce nonspecific binding of proteins to surfaces ADDIN ENRfu ***(20)***. Other additives such as BSA or serum can be used to block nonspecific binding. However, because SPR (and other optical biosensor technologies) detect all interactions at the biosensor surface, binding of blocking agents will increase the baseline signal and can sometimes lead to baseline artifacts, e.g., caused by pump pulsing. This can be avoided by using the minimal concentrations of high molecular weight additives necessary to block nonspecific binding.
11. Some cell lines, particularly murine cell lines, contain endogenous retroviruses. The presence of contaminating endogenous retroviral particles should not cause problems in biosensor assays. In fact, receptors will probably be incorporated into such particles. However, contaminating endogenous particles may lead to confusion when analyzing virus preps either by SDS-PAGE or by electron microscopy.
12. The total amount of plasmid and ratio of receptor plasmid to MLV-gag plasmid were determined empirically and will vary depending on the particular plasmids and cell types used.
13. The quality of calcium phosphate/DNA transfectant is in part determined by the mixing step. Vortexing either too slowly or too rapidly can lead to large, visible particles, which transfect poorly. A good transfection mix will look cloudy, but not contain large (visible) aggregates of precipitate.
14. Pseudotypes are sensitive to mechanical disruption. Overnight resuspension followed by gentle vortexing allows for minimal damage of particles.
15. Some binding interactions are so rapid that measurement of ligand association is limited by diffusion of ligand to the biosensor surface where receptor binding occurs. Likewise, apparent dissociation rates can be retarded by rapid rebinding of ligand. These mass transport effects can be accommodated mathematically during data analysis. Mass transport effects can also be experimentally minimized by using high flow rates and by reducing the number of ligand binding sites on the biosensor surface (few attached pseudotypes).
16. There are several programs available for global analysis of biosensor data. BIAevaluation is data analysis software provided by Biacore at http://www.biacore.com. Another program is CLAMP ***(21)***, which is available as freeware from the Protein Interaction facility at the University of Utah http://www.cores.utah.edu/interaction/.

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