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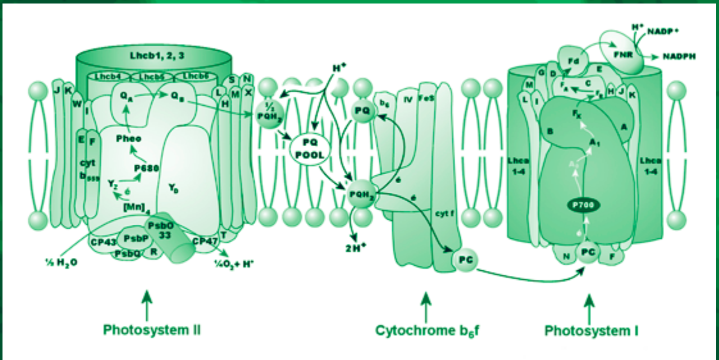

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Fractionation of Thylakoid Membranes Into Grana and Stroma Thylakoids

Juan Cuello and María José Quiles

Summary

The chloroplasts contain an extensive system of internal membranes or thylakoids in which all the light-harvesting and energy-transducing processes of the photosynthesis are located. Thylakoids are differentiated into stacked membrane regions (or grana thylakoids) and nonstacked membranes (or stroma thylakoids), each with a specialized structure and function. Both kinds of thylakoids can be separated by detergent-based methods or mechanical fragmentation such as sonication. We describe the fractionation of thylakoid membranes into grana and stroma thylakoids by treatment with the detergent digitonin and successive ultracentrifugation of the resultant vesicles. After their separation, the thylakoid fractions retain electron transport and enzymatic activities and are characterized using various parameters. The stroma thylakoids have higher chlorophyll a/chlorophyll b and protein/total chlorophyll ratios, and greater photosystem I and NADH dehydrogenase activities than the grana thylakoids. In the conditions used and on a protein basis of total thylakoids, the yield of stroma thylakoids is 5%, which is considerable taking into account that the stroma thylakoids are a minor component of total thylakoids.

Key Words: Barley; chloroplast; digitonin fractionation; grana thylakoids; *Hordeum vulgare*; stroma thylakoids; thylakoid fractionation.

1. Introduction

In photosynthetic eukaryotes, photosynthesis occurs in subcellular organelles known as chloroplasts. These are semiautonomous organelles comprising an envelope formed of two membranes, an aqueous matrix known as stroma, and an extensive system of internal membranes known as thylakoids. All of the light-harvesting and energy-transducing functions are located in the thylakoids, which form a physically continuous membrane system that encloses an aqueous compartment, the thylakoid lumen. With few exceptions, thylakoids are essentially differentiated into stacked membrane regions (grana thylakoids)

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and non-stacked membranes or membranes exposed to the stroma (stroma thylakoids) ***(1)***. Each of these two kinds of thylakoids has a specialized structure and function. Cyclic electron transport occurs in the stroma thylakoids, which account for about 20% of the thylakoid membranes, whereas linear electron transport occurs in the grana thylakoids ***(2)***. A quantitative model of the distribution of the photosynthetic components in stroma and grana thylakoids, including the three membrane domains constituting the grana thylakoids, has been proposed ***(3)***.

The first subfractionations of the thylakoid membranes used detergent-based methods, such as digitonin and Triton X-100 ***(4)***. Other methods relied on mechanical fragmentation, such as the passage of thylakoids through a French pressure cell or subjection to sonication ***(4)***. More recently, a combination of press treatment or sonication and partitioning in an aqueous polymer two-phase system or countercurrent distribution of vesicles originating from thylakoids has been exploited to isolate subthylakoid fractions ***(5,6)***. When necessary, ultrasonic disintegration of the thylakoid membranes into the grana and stroma thylakoid fractions can be used because it prevents possible partial delipidation or the enzymatic inactivations which occur when detergent-based methods are used. Digitonin has proven to be the most useful detergent for isolating vesicles of stroma thylakoids, and low amounts of this detergent appear to be selective, leaving the grana stacks mainly intact ***(4)***. Using the detergent digitonin method outlined by Leto et al. ***(7)***, we describe the fractionation of thylakoid membranes into grana and stroma thylakoids, both of which after their separation retain electron transport and enzymatic activities. With this in mind, the Methods section will be constituted by two subsections: Isolation of chloroplasts and Fractionation of thylakoid membranes into grana and stroma thylakoids.

2. Materials

1. Barley (*Hordeum vulgare L.*) leaves. Sow 25 g of barley seeds in seedling tray (30 cm × 18 cm × 5 cm) in vermiculite and water regularly with a nutrient medium (*see* **Note 1**). Grow in a plant culture chamber at 24°C under an 18 h photoperiod of white light (53 $\mu E \cdot m^{-2} \cdot s^{-1}$ or 12 $W \cdot m^{-2}$ photosynthetic active radiation). Seedlings can be harvested 8–14 d after sowing (*see* **Note 2**). For 36 g of leaves, two plantations are required, and for chloroplast isolation only the oldest leaf is used.
2. Homogenizer Sorvall Omni-mixer (for leaf samples up to 36 g) or Waring blender (for samples > 72 g).
3. Extraction buffer (E): 0.35 *M* sucrose, 25 m*M* HEPES, 2 m*M* Na_2-EDTA, 2 m*M* ascorbic acid, 4 m*M* dithiothreitol, 10 m*M* $MgCl_2$ and 1 m*M* phenylmethylsulfonyl fluoride (PMSF), pH 7.6. To make 1 liter, dissolve 119.8 g sucrose, 5.9572 g HEPES, 0.7444 g Na_2-EDTA, 0.3520 g ascorbic acid, 0.6168 g dithiothreitol, 2.0331 g $MgCl_2 \cdot 6H_2O$ and 0.1742 g PMSF in 900 mL distilled water (*see* **Note 3**). Adjust the

pH to 7.6 with NaOH and bring the volume up to 1 liter with distilled water. Store frozen in 1/3 liter aliquots until use, a maximum of 10 d after preparation. To thaw to an icy slush and keep at 0–4°C immediately before using. Discard the remaining thawed buffer if it is not used immediately for further chloroplast isolation.

4. Muslin.
5. Four 250-mL centrifuge bottles, two 50-mL and two 15-mL centrifuge tubes, and four 38-mL ultracentrifuge tubes.
6. Hypotonic buffer (H): 10 m*M* tricine, 10 m*M* NaCl and 10 m*M* $MgCl_2$, pH 7.8. To make 500 mL, dissolve 0.8960 g tricine, 0.2922 g NaCl and 1.0166 g $MgCl_2 \cdot 6H_2O$ in 450 mL distilled water. Adjust the pH to 7.8 with NaOH and bring the volume up to 500 mL with distilled water. Stored at 0–4°C, it remains stable for at least 1 mo.
7. Pure acetone.
8. 80% (v/v) acetone.
9. Buffer H containing 0.1 *M* sorbitol. To make 250 mL, dissolve 4.5550 g D-sorbitol in buffer H and bring the volume up to 250 mL. Stored at 0–4°C, it remains stable for at least 2 wk.
10. 1% (w/v) digitonin solution in buffer H containing 0.1 *M* sorbitol. To make 50 mL, dissolve at 50–60°C by heating 0.5 g of water-soluble digitonin in 45 mL of buffer H containing 0.1 *M* sorbitol and then bring the volume up to 50 mL with the same buffer. Store at 0–4°C for up to 1 wk (*see* **Note 4**).

3. Methods

The following methods describe the isolation of chloroplasts (*see* **Subheading 3.1.**) and the fractionation of their thylakoid membranes into grana and stroma thylakoids by treating with the detergent digitonin (*see* **Subheading 3.2.**). With the purpose of retaining electron transport and/or enzymatic activities in the membranous fractions obtained, all the steps of the procedures are performed at 0–4°C unless stated otherwise. Moreover, it is essential to perform the protocol quickly to obtain biochemically active fractions. Separate the chloroplasts from the homogenate as rapidly as possible.

3.1. Isolation of Chloroplasts

Chloroplasts are isolated from recently detached barley leaves by a modification of the method of Ellis *(24)*, as described by Quiles and Cuello *(25)*.

1. Harvest 36 g of leaves and wash with distilled water and then wipe with filter paper. Cut into 0.5–1.0 cm sections with scissors and place in a 400–500 mL homogenization chamber. Add 225 mL of buffer E (preferably as a semi-frozen slurry) and grind for 10 s with a Sorvall Omni-mixer homogenizer at setting 9 (14,400 rpm, according to supplier) (*see* **Notes 5** and **6**).
2. Filter the homogenate immediately through four layers of muslin into a 500-mL beaker submerged in ice. Gently squeezing the homogenate through the muslin increases chloroplast yield and saves time.

3. Divide the filtrate into two 250-mL centrifuge bottles and centrifuge at 200*g* for 5 min.
4. Carefully decant the supernatant in other two centrifuge bottles and centrifuge it at 2500*g* for 10 min to precipitate the chloroplasts.
5. Discard the supernatant by careful decantation, hold the bottles upside down and wipe insides with filter paper. Continuously wash the pellets of crude chloroplasts to reduce cytoplasmic contamination by resuspending the pellets gently in 40 mL buffer E (20 mL for every pellet) using a paintbrush until the suspension appears uniform. Transfer both chloroplast suspensions to one 50-mL centrifuge tube.
6. Centrifuge at 2500*g* for 10 min.
7. After discarding the supernatant, repeat the chloroplast washing by gently resuspending the pellet in 40 mL buffer E using a paintbrush and centrifuge again at 2500*g* for 10 min. The pellet is constituted by the washed chloroplasts (*see* **Note 7**), which are used immediately to isolate grana and stroma thylakoids.

3.2. Fractionation of Thylakoid Membranes Into Grana and Stroma Thylakoids

Thylakoid membranes were fractionated into grana and stroma thylakoids essentially as described by Leto et al. *(7)*.

1. Resuspend the washed chloroplast pellet obtained in **step 7** of **Subheading 3.1.** in 45 mL buffer H (to osmotically break the chloroplasts) with gentle shaking until a uniform suspension is obtained, and centrifuge at 2500*g* for 10 min.
2. Discard the supernatant containing the chloroplast stroma and wash the pellet of total chloroplast membranes. To do this, resuspend the pellet in 45 mL of buffer H with gentle shaking, and centrifuge again at 2500*g* for 10 min.
3. Discard the supernatant and resuspend the pellet of washed chloroplast membranes in 7 mL of buffer H containing 0.1 *M* sorbitol with gentle shaking until a homogeneous suspension is obtained, before measuring the exact final volume of suspension.
4. Determine the chlorophyll concentration of the previous suspension by taking 0.05 mL and mixing with 0.95 mL distilled water plus 4 mL pure acetone in a 15 mL centrifuge tube (make a duplicate mixture to calculate the mean value). Vortex the mixture and then centrifuge for 5 min at 2500*g*. The absorbances at 646 and 663nm in the supernatant are determined using 80% (v/v) acetone as a blank; calculate the concentration using the Lichtenthaler and Wellburn formula *(8)*:

   $$\text{Total chlorophyll (mg/L)} = 17.32\ A_{646} + 7.18\ A_{663}$$ (*see* **Note 8**)

   Thus, the chlorophyll concentration (mg/mL) in the suspension of **step 3** is obtained by dividing the value given by this formula by 10. This concentration must be at least 0.8 mg chlorophyll/mL. This whole step must be carried out at room temperature.
5. Bearing in mind the chlorophyll concentration obtained in the suspension of **step 3**, add buffer H containing 0.1 *M* sorbitol and 1% digitonin solution to final concentrations of 0.4 mg chlorophyll/mL and 0.5% (w/v) digitonin in the suspension (*see* **Note 9**).

Table 1
Chlorophyll a/Chlorophyll b and Protein/Total Chlorophyll Ratios and NADH-FeCNR and Photosystem Activities of the Grana and Stroma Thylakoids

Parameter	Thylakoid membranes	Grana thylakoids	Stroma thylakoids
Chl a/b (w/w)	4.2	3.6	10.6
Protein/total Chl (w/w)	5.5	4.8	6.0
NADH-FeCNR (U (mg protein)$^{-1}$)	0.11 ± 0.01	0.06 ± 0.01	0.41 ± 0.05
PSI (U (mg Chl)$^{-1}$)	0.43 ± 0.08	0.25 ± 0.06	0.70 ± 0.04
PSII (U (mg Chl)$^{-1}$)	0.35 ± 0.04	0.49 ± 0.03	0.02 ± 0

The values are means ± SE from three to five independent experiments. Chl, chlorophyll. U, unit of activity. Data from Quiles et al. ***(12)***.

6. Incubate at 0–4°C for 30 min with gentle and continuous shaking in the dark.
7. Centrifuge the resulting suspension at 1000*g* for 3 min to remove the undigested material.
8. Discard the pellet and decant the supernatant containing the thylakoid membranes solubilized with digitonin into two ultracentrifuge tubes. Ultracentrifuge at 40,000*g* for 30 min.
9. Decant the green supernatants containing the stroma thylakoids into one ultracentrifuge tube and resuspend the pellets of grana thylakoids in 18 mL (9 mL for every pellet) of buffer H. Store the suspension of grana thylakoids at –20°C until use (*see* **Note 10**).
10. Ultracentrifuge the supernatant containing the stroma thylakoids at 144,000*g* for 90 min to precipitate the stroma thylakoids.
11. Discard the supernatant and resuspend the pellet of stroma thylakoids in 1.5 mL of buffer H. Store at –20°C until use (*see* **Notes 11** and **12**).

As previously described ***(9–12)***, grana and stroma thylakoids can be characterized by using various parameters, including chlorophyll a/chlorophyll b ratio, protein/total chlorophyll ratio and photosystem (PS) I and II activities. **Table 1** shows the values of these parameters and those of NADH-ferricyanide oxidoreductase (NADH-FeCNR) activities for the grana and stroma thylakoids obtained using the described protocol. The chlorophyll a/chlorophyll b ratio is approximately three times greater in the stroma than in the grana thylakoids, in accordance with the findings of other authors ***(9–11)***. As a result of the enrichment of the ATP synthase complex in the stroma thylakoids ***(10)***, the stroma thylakoids contain more protein per unit of chlorophyll than the grana thylakoids. The stroma thylakoid fraction, which lacks PSII activity, is very rich in PSI activity, as observed by Ford et al. ***(9)***. **Table 1** shows that the NADH-FeCNR activity associated to the thylakoid NADH dehydrogenase complex ***(20,25)***, is seven times greater in stroma than in grana thylakoids. This reflects the fact that the chloroplast NADH dehydrogenase complex, which is NADH-specific ***(13,14)***, is preferentially located in stroma thylakoids ***(12,14)***. Moreover, both thylakoid fractions retain NADPH-FeCNR activity, probably owing to the diaphorase activity of ferredoxin-NADP oxidoreductase ***(12)***.

4. Notes

1. Method can be used with any nutrient medium. If Crone medium is used, water each seedling tray with 2 g KNO_3, 1 g $CaSO_4{\cdot}2H_2O$, 1.58 g $MgSO_4{\cdot}7H_2O$, 0.5 g $Ca_3(PO_4)_2$ and 0.5 g $Fe_3(PO_4)_2{\cdot}8H_2O$ in 2 L of tap water and, if necessary, only tap water. All these salts must be of low purity grade.
2. To prevent the chloroplasts from breaking during centrifugation, the leaves must be free from starch grains ***(15)***. The plants must be grown under a lower light intensity (20 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$) during the last photoperiod or in dark during the last 24 h. However, this last low light or dark treatment may affect the electron transport activity of the isolated thylakoids because the thylakoid NADH dehydrogenase complex mediates cyclic electron transport around PSI in light ***(16,17)*** and PSI and NADH dehydrogenase complex activities have been found to increase with light ***(18,19)***.
3. The extraction buffer should contain an osmoticum, such as 0.35 *M* sucrose, to prevent osmotic breakage of the chloroplasts during the extraction process and a protease inhibitor, such as PMSF, to minimize the hydrolytic effect of the proteases released from the vacuole during homogenization. Before dissolving the sucrose, dissolve the rest of the buffer components, especially the PMSF, which is the least soluble product.
4. Use water-soluble digitonin, which can be supplied by SIGMA. This product is highly toxic. To handle, wear suitable protective clothing, gloves, and eye/face protection. Do not breathe the dust.
5. Starting with 72 g or more of leaves, add (number g of leaves × 6.25) mL of buffer E and homogenize in a Waring blender operating at the lowest speed (15,000 rpm) with three pulses of 4 s.
6. The chloroplast yield depends on the degree of leaf grinding, and thus on the sharpness of the homogenizer blades. These must be suitably sharpened.
7. Use enzymatic markers and compare the polypeptide profiles of mitochondria and chloroplast to determine if this chloroplast preparation is essentially free from other cellular components ***(20)***.
8. For a chlorophyll solution in 80% (v/v) acetone, the total chlorophyll concentration can also be obtained by the classical Arnon formula ***(21)***, which gives a higher value than that obtained by the Lichtenthaler and Wellburn formula by a factor of 1.11 ***(22)***.
9. If digitonin begins to precipitate in the 1% (w/v) digitonin solution in buffer H containing 0.1 *M* sorbitol, redissolve before using by heating at 50–60°C for several min before cooling again to 0–4°C.
10. The grana thylakoids obtained are a crude fraction. To obtain a highly enriched grana fraction, and if enzymatic inactivation during the isolation procedure is not considered very important, continue as described by Leto et al. ***(7)***:

   a. Resuspend the crude grana thylakoid pellet to reach 1.5 mg chlorophyll/mL in buffer H containing 0.1 *M* sorbitol and 0.5% (w/v) digitonin, and add Triton X-100 with vortex mixing to reach a final Triton/chlorophyll ratio of 15/1 (w/w).
   b. One min after mixing, dilute the granal suspension with buffer H containing 0.1 *M* sorbitol and 0.5% digitonin, and centrifuge at 1000*g* for 3 min to remove the pellet of undigested material.
   c. Centrifuge the supernatant at 40,000*g* for 30 min to obtain the pellet of grana thylakoids, which is resuspended in buffer H.

11. Compared with the total thylakoid membranes obtained with the protocol described, the yield of stroma thylakoids is 3.6 ± 0.3% and 4.9 ± 0.3% (w/w) (means ± SE, n = 5) based on chlorophyll and protein, respectively (results obtained in our laboratory), which is considerable taking into account that, as indicated in the Introduction, the relative content of stroma thylakoids in chloroplasts is low.
12. To prevent possible partial electron transport and/or enzymatic inactivation caused by digitonin, stroma thylakoids can be obtained by sonication from **step 2** of **Subheading 3.2.** following the method of Ford et al. ***(9)*** with modifications, as described by Quiles et al. ***(23)***:
    a. Resuspend the pellet of washed chloroplast membranes in a buffer 5 m*M* potassium phosphate, 130 m*M* KCl, 0.2 *M* sorbitol, pH 7.5, at 0.4 mg chlorophyll/mL.
    b. Incubate for 45 min at 0–4°C in the dark with occasional stirring.
    c. Sonicate the thylakoid suspension in a Dynatech Sonic Dismembrator, Model 300 (ARTEK) at 50% intensity for three pulses of 30 s each with 60 s resting intervals and continuous cooling.
    d. Ultracentrifuge the sonicate at 25,000*g* for 30 min.
    e. Ultracentrifuge the supernatant at 40,000*g* for 1 h. Resuspend the pellet of stroma thylakoids obtained in 1.5 mL of buffer 5 m*M* potassium phosphate, 130 m*M* KCl, 0.2 *M* sorbitol, pH 7.5. Store at –20°C until use. The stroma thylakoids isolated through sonication have high chlorophyll a/chlorophyll b and protein/total chlorophyll ratios and high PSI and NADH-FeCNR activities ***(23)***. However, from total thylakoid membranes of 8-d old barley leaves, the method yields only 2.1 ± 0.3% and 2.3 ± 0.3% (w/w) (means ± SE, *n* = 5) based on chlorophyll and protein, respectively (results obtained in our laboratory), percentages which are lower than those obtained using the digitonin-based method.

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2

Isolation of Photosystem I Particles From Spinach

Tetsuo Hiyama

Summary

A method to prepare photosystem I (PSI) particles is described. Spinach leaves are used to prepare broken chloroplasts that are then solubilized by using a detergent (Triton X-100). Solubilized chloroplasts are then applied on an ion-exchange column. Eluted by a linear concentration gradient of NaCl, fractions enriched in PSI particles are collected and applied on a small hydroxyapatite column. By eluting with phosphate buffer, a concentrated preparation of PSI particles is obtained. The particles consist of PsaA, PsaB, PsaC, PsaD, PsaE, and PsaY. Assay methods that involve SDS-PAGE and P700 determination are also presented.

Key Words: Photosystem I; preparation; reaction center.

1. Introduction

Functionally, photosystem I (PSI) is defined as “a pigment–protein complex” embedded in thylakoid membranes that can photoreduce ferredoxin by electrons from photosystem II (PSII) fed through plastocyanin ***(1)***. In short, it may also be called a “light-driven plastocyanin: ferredoxin oxidoreductase”, although its inherently irreversible nature might not fit well the word “oxidoreductase” in its enzymological sense. The core of the complex is a heterodimer of two, 80 kDa polypeptides (PsaA and PsaB). This core binds a P700 (the photochemical reaction center pigment: a heterodimer of chlorophylls *a* and *a*'), two phylloquinones, an iron-sulfur cluster and a number of light-harvesting chlorophyll *a* molecules. Thus far, as many as 15 other subunits smaller than 20 kDa have been proposed to be members of the PSI complex.

Efforts to isolate PSI activity in a form of a complex date back to the 1960s. Currently, a variety of different preparations have been reported from numerous photosynthetic organisms. Their subunit compositions vary widely even within the same plant species. Those complexes are categorized into three types:

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Types I, II, and III *(1)*. One of the most common types of PSI complex, categorized as Type II, consists of PsaA, PsaB, PsaC, PsaD, PsaE, and occasionally a few other small polypeptides. Core complexes that consist only of large subunits (PsaA and PsaB) are Type III. This chapter discusses Type II preparation; Type III will be discussed in Chapter 6.

2. Materials

1. Spinach leaves (*see* **Note 1**).
2. Kitchen blender.
3. Cheese cloth on a funnel (for filtration).
4. Chloroplast preparation buffer: 50 m*M* sodium phosphate buffer, pH 7.0, and 10 m*M* NaCl.
5. Centrifuge, refrigerated type.
6. Spectrophotometer.
7. Temperature-controlled water bath (45°C and 37°C).
8. Solubilization medium: 50 m*M* Tris-HCl, pH 8.8, and 3% Triton X-100 (*see* **Note 2**).
9. Column chromatography apparatus equipped with a peristaltic pump, a gradient maker (**Note 3**), a three-way valve, and a fraction collector (*see* **Note 4**).
10. Anion-exchange column (BioRad Econo-Pac HighQ, 5-mL type).
11. Starting buffer: 10 m*M* Tris-HCl, pH 8.8, 0.2% Triton X-100, and 20% sucrose.
12. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus with a gradient former. Less time-consuming mini-size (10 cm × 10 cm) equipment is preferred for quick analysis.
13. Pretreatment medium: 90 m*M* Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, and 0.6 *M* sucrose.
14. Upper and lower electrode buffer: 25 m*M* Tris-hydroxyaminomethane (Tris), 192 m*M* glycine, and adjust 0.1% SDS to pH 8.3 at room temperature. Supplement the upper buffer with 0.1 mL/100 mL of 0.1% ethanol solution of bromophenolblue (BPB).
15. Stacking gel: 4.87% acrylamide, 0.13% methylene-*bis*-acrylamide, 0.125 *M* Tris-HCl buffer, pH 6.8, and 7.5 *M* urea.
16. Separating gel: linear gradient of the following monomer solutions (a and b): a, 16% acrylamide, 0.27% methylene-*bis*-acrylamide, 7.5 *M* urea and 600 m*M* Tris-HCl, pH 8.8; b, 22% acrylamide, 0.37% methylene-*bis*-acrylamide, 6% sucrose, 7.5 *M* urea and 600 m*M* Tris-HCl, pH 8.8.
17. Staining solution: 0.23% Coomasie brilliant blue (CBB) in 50% methanol and 10% acetic acid.
18. Hydroxyapatite medium: 10 m*M* Tris-HCl, pH 7.5, and 0.6 m*M* $CaCl_2$.
19. Hydroxyapatite column (BioRad Econo-Pac CHT-II, 1-mL type).
20. Equilibration buffer: 10 m*M* Tris-HCl, pH 8.8, 0.3 m*M* $CaCl_2$, and 0.05% Triton X-100.
21. Elution buffer: 50 m*M* sodium phosphate buffer, pH 8.0 and 0.05% Triton X-100.
22. Deep freezer (below –50°C, preferably –80°C).

3. Methods

3.1. Preparation

Preparation should be conducted at low temperatures (approx 4°C). The methods described below outline ***(1)*** the preparation of broken chloroplasts, solubilization, and column chromatographies, ***(2)*** assay methods including SDS-PAGE, optical measurement of chlorophyll concentration, and optional P700 determination.

3.1.1. Preparation of Broken Chloroplasts

1. Spinach leaves, remove ribs and wash in ice water, loosely packing in plastic bags, and leave overnight in a cold room (*see* **Note 5**).
2. Blend leaves (about 100 g wet weight) in a kitchen mixer with 500 mL of chloroplast preparation buffer. Thirty seconds is usually adequate.
3. Combine two batches (about 1000 mL from 200 g leaves) and filter through four layers of cheesecloth.
4. Centrifuge the filtrate 10,000*g* for 5 min. Resuspend the precipitate in the same buffer.
5. Adjust the chlorophyll concentration by dilution to 2 mg/mL. For this purpose, a rough estimation of total chlorophylls is adequate (*see* **Note 6**).

3.1.2. Solubilization

1. Mix one volume of the suspension with two volumes of preheated (45°C) solubilization medium at the chlorophyll concentration of 2 mg/mL and incubate for 30 min at 45°C (*see* **Note 7**).
2. Chill the suspension (approx 50 mg chlorophylls) in an ice bath and centrifuge at 12,000*g* for 30 min to remove debris.
3. Collect the supernatant and use in the next step.

3.1.3. Column Chromatography

1. Wash a newly purchased HighQ column with 100 mL of the starting buffer (*see* **Note 8**).
2. Keep the flow rate constant at 1 mL/min throughout the procedure.
3. The supernatant (approx 50 mg chlorophylls) is loaded on the column by injecting it through the three-way valve (*see* **Note 4**).
4. Wash the loaded column with 1000 mL of the starting buffer supplemented with 10 m*M* NaCl, then with 300 mL of the starting medium supplemented with 50 m*M* NaCl, and finally with 400 mL of linear gradient NaCl (50–200 m*M*, *see* **Note 3**). The concentration gradient is formed by filling a mixing chamber of the gradient apparatus with 200 mL of the starting buffer supplemented with 50 m*M* NaCl and the bottom-connected chamber with an equal volume of the same buffer supplemented with 200 m*M* NaCl.

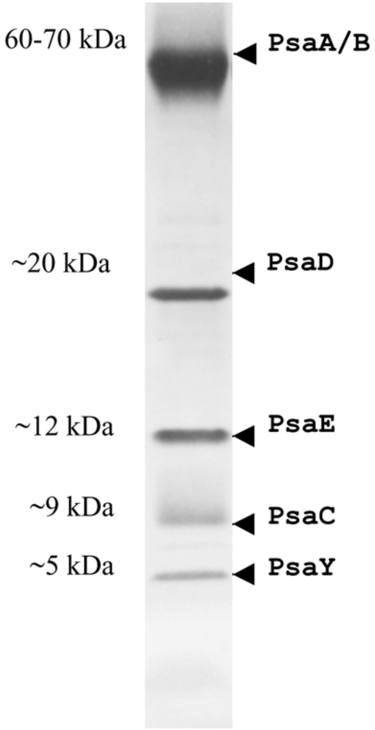

Fig. 1. SDS-PAGE of Type II PSI particles from spinach. Electrophoresis was performed as described in the text.

5. Subject collected fractions (1 mL each) to SDS-PAGE for PSI assay (*see* **Note 8**). Collect and dilute fractions that show a typical PSI pattern (**Fig. 1**) with an equal volume of the hydroxyapatite medium, and then load on an Econo-Pac CHT-II column equilibrated with the equilibration buffer. To load, inject the combined fractions through a 50-mL syringe directly fitted on the column.
6. Wash the column with 20 mL of the above medium, then elute with a small volume (1–2 mL) of the elution buffer to obtain a concentrated preparation.
7. Immediately supplement this final preparation with 20% sucrose for stabilization and store in a deep freezer for months.

3.2. Assays

3.2.1. SDS-PAGE

For SDS-PAGE, a linear gradient gel supplemented with urea gives a favorable result. We routinely use a mini-size slab gel (10 cm wide and 7 cm high for separating gel and 1.5 cm high for stacking gel on top; 1 mm thick).

1. Mix a sample suspension with an equal volume of a pretreatment medium, and incubate at 37°C for 20 min before applying on the gel.
2. Prepare the electrophoresis gel using the stacking and separating gel solutions.
3. Perform electrophoresis at room temperature using upper and lower buffers.
4. Apply 70 volt until the BPB front line reaches the stacking gel, then raise the voltage to 170 volt.
5. Stop electrophoresis when the BPB front line comes near the end.
6. Stain the gel for 30 min with the staining solution, and then rinse in 7% acetic acid for destaining.

3.2.2. Optical Measurements

Absorbance is measured by using a spectrophotometer for determination of chlorophyll concentration (*see* **Note 6**). Any type of spectrophotometer can be used for this purpose.

3.2.3. P700 Determination

This step is optional because the method requires specialized equipment (*see* **Note 9**).

4. Notes

1. Spinach (*Spinacia oleracea*) available at markets comes in a wide variety of cultivated strains. They not only differ from locality to locality but also depend on seasons. Fortunately for broken chloroplast preparation, most varieties of spinach, in any season, can be successfully used for this purpose. Preparation yields may fluctuate somewhat.
2. The pH values of the media and buffers stated in this chapter are all measured and adjusted at room temperatures.
3. Any type gradient maker can be used. An apparatus routinely used in our laboratory consists of a mixing chamber (cylinder) with two spouts at the bottom. One spout is connected to another chamber of the same size and shape, with a short silicon rubber tubing pinched by a pinch cock. The mixing chamber is filled with a medium with a lower salt concentration and the other with a higher concentration medium. The mixing chamber with a stirring bar on the bottom is placed on a magnetic stirrer. Before starting the flow, the pinch cock is removed and the stirring is initiated. A device of the similar but smaller type is used for gradient gel making.
4. Prepacked columns with an assembly based on luer fittings are recommended. Utilizing a peristaltic pump between the column and a buffer reservoir, flow rate can be kept constant. A simple three-way valve (a disposable luer fitting type) is inserted between the pump and the column for sample injection.
5. This overnight cold storage is rather optional; washed leaves can be used immediately without much trouble.

6. A rough molar extinction coefficient (90 mg^{-1} mL^{1} cm^{-1} or 100 mM^{-1} cm^{-1}) at 665 nm for chlorophyll *a* in 80% acetone can be used for this calculation. Add 3 mL of the chloroplast suspension to 3 mL of 80% acetone and mix well. Measure the absorbance at 665 nm (A_{665}). A rough chlorophyll *a* concentration is calculated as A_{665} × 1000/90 (mg/mL). Here, chlorophyll means chlorophyll *a*, as chlorophyll *b* content in PSI is low.
7. Heat treatment is quite effective to remove PSII and other heat-labile components.
8. The column can be rejuvenated by washing with the starting buffer supplemented with 1.0 *M* NaCl, and used repeatedly several times.
9. The best way to prepare photochemically sound particles is to collect fractions enriched in the reaction center pigment P700. P700 can be determined in several ways: light-induced oxidation, chemical oxidation, and determination of chlorophyll *a*'. For the oxidation methods, either light induced or chemically induced, it is essential to use a spectrophotometer highly sensitive and stable enough to measure absorbance changes as small as 0.001. Preferably, a photodetector (e.g., photomultiplier) is set near the cuvet so that scattering interference may be minimal. Such instruments currently available include a Shimadzu MPS-2400 spectrophotometer. I recommend the chemical method, because a custom-made accessory for either flash or continuous illumination is needed for measurement of light-induced changes ***(2,3)***. The chemical procedure is as follows:
    a. Add 10 μL each of 0.1 m*M* TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) and 0.1 m*M* potassium ferricyanide to 3 mL of the reaction buffer (50 m*M* Tris-HCl, pH 8.8 supplemented with 0.05% Triton X-100) in a standard cuvet (1-cm light path), and mix well.
    b. Scan and record the absorbance spectrum from 650 nm to 750 nm. It is essential to store the data in a computer memory.
    c. Then, add 10 μL of 2 m*M* ascorbic acid to the same cuvet using a disposable tiny-headed coffee spoon, and stir it well without disturbing the cuvet position.
    d. Scan again, and subtract the spectrum recorded prior to obtain a difference spectrum (oxidized-minus-reduced). From the trough size around 700 nm, the concentration of P700 can be calculated by using a molar extinction coefficient for P700, 64 mM^{-1} cm^{-1} ***(4)***.

The 3-D structure analysis of the PSI reaction center ***(5)*** determined that the molecular nature of P700 is a heterodimer of chlorophyll *a* and chlorophyll *a*' ***(6,7)***. The determination of chlorophyll *a*' involves a special extraction procedure followed by a high-performance liquid chromatography (HPLC) analysis, beyond the scope of this chapter. Refer to Maeda and colleagues for details ***(8).***

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3

Rapid Isolation and Purification of Photosystem I Chlorophyll-Binding Protein From *Chlamydomonas reinhardtii*

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Summary

The available procedures for isolation and purification of photosystem I (PSI) from *Chlamydomonas reinhardtii* are time consuming and usually require several hours of sucrose gradient ultracentrifugation steps. This may lead to structural and functional impairment, including release of pigments and/or dissociation of protein subunits. Moreover, it is difficult to isolate intact complexes from thylakoids containing mutated PSI that accumulate to lower levels. Hence, isolation of intact PSI core complex depends on the speed of the procedure and the mildness of the extraction and purification. We have, therefore, modified the procedure for PSI isolation to both increase the yield of PSI and to reduce contamination by other pigment protein complexes. The modified procedure involves dodecyl maltoside solubilization of crude-thylakoid membranes followed by single-step column chromatography using a weak anion-exchanger. PSI eluted from the column between 13 m*M* and 15 m*M* Mg $S0_4$. This new rapid purification procedure yielded pure PSI preparations with a Chl/P700 ratio of approx 90 and showing typical absorption difference spectra with a maximum bleaching occurring at 696 nm. Femtosecond transient absorption spectroscopy of purified PSI complex revealed a high degree of similarity in terms of excitation energy transfer within the PSI core to observations in cyanobacterial PSI.

Key Words: *Chlamydomonas reinhardtii;* core complex; dodecyl maltoside; green alga; ion exchange chromatography; membrane protein purification; photosystem IChl/P700.

1. Introduction

Photosystem I (PSI) is a multi-subunit pigment protein complex embedded in the thylakoid membrane of cyanobacteria, algae, and plants. It mediates the light-driven electron transport from plastocyanin or cytochrome C_6 to ferredoxin. Although eukaryotic PSI has many similarities to cyanobacterial PSI, both in structure and in function, there are also important differences. Eukaryotic PSI

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is composed of a core complex and a light-harvesting complex (LHC). The green unicellular alga *Chlamydomonas reinhardtii* has emerged as a powerful model organism for genetic and biochemical/biophysical analysis of photosynthesis ***(1)***. Photosynthetic function is dispensable in this alga and mutants deficient in photosynthetic activity can be grown in media supplemented with acetate. The establishment of reliable chloroplast and nuclear transformation methods for *Chlamydomonas reinhardtii* has opened the door for an in-depth analysis of the structure-function relationship of photosynthetic complexes ***(1)***. However, the available procedures for isolation and purification of PSI from this alga are time consuming and usually require several hours of sucrose gradient ultra centrifugation steps. This may lead to structural and functional impairment, including release of pigments and/or dissociation of protein subunits ***(2)***. Moreover, it is difficult to isolate intact complexes from thylakoids containing mutated PSI that accumulate to lower levels. Hence, isolation of intact PSI core complex depends on the speed that the procedure is conducted and the mildness of the extraction and purification. Therefore, we have modified the procedure for PSI isolation from green alga *C. reinhardtii* to both increase the yield of PSI and to reduce contamination by other pigment protein complexes. The modified procedure involves dodecyl maltoside solubilization of crude thylakoid membranes followed by single-step column chromatography using a weak anion-exchanger.

2. Materials

1. Strains: *Chlamydomonas reinhardtii* strains CC125 and CC2696.
2. Medium : 1× Cox *Chlamydomonas* medium (CC): 2.5 g Tris-HCl base, 1 mL glacial acetic acid, 0.5 g NH_4NO_3, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$, 0.05 g KH_2PO_4, 0.1 g KCl, 1 mL Hunter's trace elements solution.
3. Add yeast extract to the liquid CC medium (1X) at a final concentration of 0.1% before autoclave.
4. Hutner's trace element solution: For 1L of trace elements mix, dissolve each compound in the volume of water indicated. Dissolve EDTA in boiling water, and prepare the $FeSO_4$ last to avoid oxidation. EDTA, disodium salt (50 g in 250 mL H_2O), $ZnSO_4$. H_2O (22 g in 100 mL H_2O), H_3BO_3 (11.4 g in 200 mL H_2O), $MnCl_2{\cdot}4H_2O$ (5.06 g in 50 mL H_2O), $CoCl_2{\cdot}6H_2O$ (1.61 g in 50 mL H_2O), $CuSO_4{\cdot}5H_2O$ (1.57 g in 50 mL H_2O), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1.1 g in 50 mL H_2O), $FeSO_4$.7 H_2O (4.99 g in 50 mL water).

   Mix all solutions except EDTA. Bring this mixture to a boil and add EDTA. The mixture should turn green. When everything is dissolved, cool to 70°C. Keeping the temperature at 70°C, adjust the pH to 6.7 with 80–90 mL hot KOH (20%). Bring the final solution to 1 L total volume. It should be clear green initially. Stopper the flask with a cotton plug and let it stand for 1–2 wk, shaking it once a day. Filter the solution using two layers of Whatman no.1 filter paper. When the solution turns purple it can be stored in the refrigerator.

5. Buffer A (breaking buffer): 50 mm HEPES, pH 7.2, 5 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 20% glycerol (v/v).
6. Buffer B: 50 mm HEPES pH 7.2, 5 m*M* $MgCl_2$, 12 m*M* $CaCl_2$, 20% glycerol (v/v).
7. Buffer C: 50 mm HEPES pH 7.2, 5 m*M* $MgCl_2$, 12 m*M* $CaCl_2$, 20% glycerol (v/v), 100 m*M* $MgSO_4$.
8. Buffer D: 50 mm HEPES, pH 7.2, 5 m*M* $MgCl_2$, 12 m*M* $CaCl_2$, 0.03% β-D-maltoside, 5 m*M* sodium ascorbate, 10 μ*M* phenazine methosulfate.
9. Protease inhibitors:1 m*M* benzamidine, 1 m*M* phenyl methyl sulfonyl fluoride (PMSF) and 1 m*M* EDTA.
10. French press.
11. Detergent: *n*-dodecyl-β-D-maltoside (Sigma).
12. Chromatography equipment: Column (26 × 2.7 cm).
13. Peristaltic pump (Model P-3, Pharmacia).
14. DEAE Toyopearl 650 S (weak anion exchanger, Toso Hass, Japan).
15. Gradient mixer GM-1 (Pharmacia).
16. Fraction collector (RediFrac, Pharmacia).
17. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
18. 1% SDS sample buffer.
19. Ultra filters: Centricon 100 (Amicon).

3. Methods

The methods described below outline:

1. The strains and culture conditions.
2. Preparation of thylakoid membrane.
3. Extraction and purification of the PSI core complex by ion exchange column chromatography.
4. Analysis and characterization of the PSI core complex proteins (*see* **Note 1**).

3.1. Strains and Culture Conditions

1. *Chlamydomonas reinhardtii* strains Wt CC 125 mt^+ and Wt CC 2696 were obtained from the *Chlamydomonas* culture collection at Duke University. Strain CC 2696 was used to prepare the PSI without contamination by PSII and peripheral *Chlb*-containing antenna complex, as CC2696 strain carries the DS521 cab deficiency mutation and a *psbA* deletion mutation.
2. Cells were maintained on 1.5% agar plates containing Cox *Chlamydomonas* medium (CC).
3. For thylakoid membrane preparation, starter culture was initiated by growing the cells heterotrophically in 250 mL flasks containing autoclaved 100 mL CC liquid medium at 25°C under the light intensity of 50 μm photons m^{-2} s^{-1} with constant shaking for 3–4 d according to Harris *(3)*.
4. Then 1–2 mL of starter culture was transferred to 2 L flasks containing sterilized 1000 mL of CC medium and grown for 5 d under the same condition as described above. **Figure 1** indicates schematically the isolation procedure for obtaining the PSI core complex.

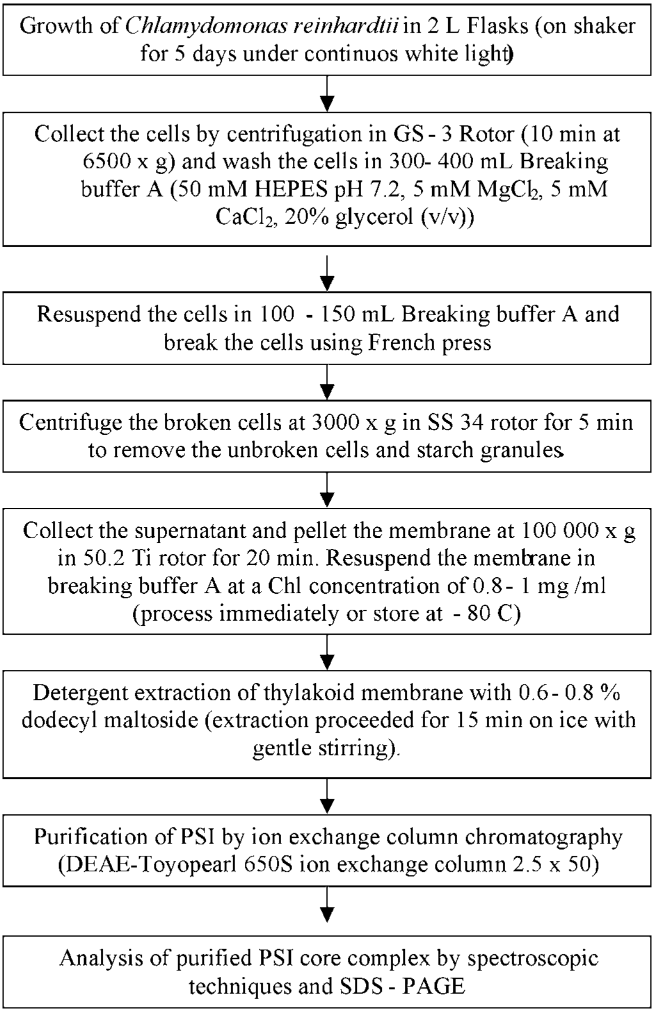

Fig. 1. Flow diagram of the preparation of thylakoid membranes and purification of PSI core complex from *Chlamydomonas reinhardtii*.

3.2. Preparation of Thylakoid Membrane

1. Cells from 6 L of medium were harvested during exponential growth at a density of 2×10^6 cells/mL by centrifugation at 4°C for 10 min at 6000*g* in a Sorvall GS-3 rotor.
2. The pellets were suspended in 350 mL of buffer A and centrifuged for 15 min at 9000*g* in Sorvall GS3 rotor.
3. The resulting pellets were resuspended in 125 mL of same buffer A containing 1 m*M* Benzamidine, 1 m*M* phenyl methyl sulfonyl fluoride (PMSF) and 1 m*M* EDTA at 2×10^8 cells/mL.

4. Cells were broken in chilled French press at 660 kg/cm^2 pressure.
5. Unbroken cells and starch granules were removed from the membrane suspension by centrifugation for 5 min at 2000*g* in a Sorvall SS-34 rotor.
6. Crude thylakoid membranes in the supernatant were pelleted by centrifugation at 100,000*g* for 20 min in a Beckman 50.2 Ti rotor at 4°C.

3.3. Extraction and Purification of the PSI Core Complex by Ion Exchange Column Chromatography (see Note 2)

1. The membrane pellets were washed once with buffer A containing 1 m*M* benzamidine, 1 m*M* phenyl methyl sulfonyl fluoride (PMSF) and 1 m*M* EDTA and resuspended in the same buffer A at a Chlorophyll (Chl) concentration of 0.8–1 mg/mL.
2. A 10% stock solution of dodecyl maltoside (Sigma) was added drop wise to this suspension of thylakoid membranes for a final concentration of 0.6% detergent.
3. Extraction proceeded in the dark for 15–20 min on ice with gentle stirring.
4. The suspension was then centrifuged at 125,000*g* in a Beckman 50.2 Ti rotor for 20 min at 4°C.
5. The supernatant was loaded onto a DEAE Toyopearl 650 S column (2.7 × 26 cm, a weak anion exchanger, Toso Haas) previously equilibrated with 500 mL of buffer A containing 1 m*M* benzamidine, 1 m*M* PMSF and 1 m*M* EDTA + 0.03% dodecyl maltoside (*see* **Note 3**).
6. The column was washed successively with 600 mL of buffer A containing 1 m*M* benzamidine, 1 m*M* PMSF and 1 m*M* EDTA + 0.03% dodecyl maltoside and with 500–600 mL of buffer B containing 1 m*M* benzamidine, 1 m*M* PMSF and 1 m*M* EDTA + 0.03% dodecyl maltoside and then with 150 mL of 5 m*M* $MgSO_4$ in buffer B at a flow rate of 6–7 mL/min using a peristaltic pump (Model P-3, Pharmacia) (*see* **Note 4**).
7. The wash with buffer A, buffer B, and 5 m*M* $MgSO_4$ in buffer B elutes the free pigments successively, mainly the carotenoids (*see* **Note 5**).
8. Then a 500 mL linear gradient from 5 to 25 m*M* $MgSO_4$ in buffer B was applied to the column (*see* **Note 6**).
9. Fractions were collected in 3–5 mL each using a fraction collector (RediFrac, Pharmacia).
10. The eluted fractions were scanned between 400 and 800 nm in a Shimadzu ultraviolet 160 spectrophotometer.
11. Fractions with Chl absorbance maxima between 675 and 677 nm were pooled and concentrated to the desired concentration using a Centricon 100 (Amicon) at 3000*g* in a Sorvall SS-34 rotor at 4°C.
12. **Table 1** shows the yield and electron transfer activity of purified PSI by ion exchange column chromatography.

3.4. Analysis and Characterization of PSI Core Complex Proteins

3.4.1. Chlorophyll Assay

1. The chlorophyll concentration in the thylakoid membrane/purified PSI was determined by adding 20 or 10 μL of respective sample to 80% of acetone.

Table 1
Oxygen Uptake and Chlorophyll Yield During Isolation of PSI From *Chlamydomonas Reinhardtii*

Material	O_2 uptake µM O_2/mg chl/h	Total Chl mg Chl	Total yield (%)
Cells	275	60.00	100.0
Thylakoids	255	50.00	83.0
DM extracts	260	39.00	65.0
PSI from column	1280	02.35	03.9

2. This solution was mixed by inverting three to six times, and centrifuging for 2 min at room temperature in a microfuge.
3. Chlorophyll concentration in the supernatant was determined by measuring absorption with a spectrophotometer at 645 and 663 nm using the following equation *(4)*:

$$\text{Chlorophyll } b \ (\mu\text{g/mL}) = 22.9 \times A_{645}) - (4.68 \times A_{663});$$
$$\text{chlorophyll } a \ (\mu\text{g/mL}) = 12.7 \times A_{663}\ ) - (2.69 \times A_{645});$$
$$\text{Total chlorophyll} = \text{Chl } a + \text{Chl } b = (20.2 \times A_{645}) + (8.02 \times A_{663}).$$

3.4.2. Room Temperature Absorption Spectrum

The room temperature absorption spectrum of purified PSI core complex was recorded on a spectrophotometer between 400 and 750 nm and is illustrated in **Fig. 2**. An absorbance maximum was observed at 677 nm. This is a typical absorbance spectrum of a PSI core complex, and is nearly identical to the PSI core complex from *Synechocystis sp.* and spinach. The spectra indicate that a large fraction of the carotenoid, absorbing between 450 and 500 nm, was removed from this preparation.

3.4.3. Flash-Induced Absorption Spectrum

1. The flash-induced absorption spectra (**Fig. 3**) were recorded as described by Webber et al. *(5)* using a laboratory-built flash spectrophotometer.
2. The measuring light from a 55 W tungsten halogen lamp was passed through a monochromator with 3 nm bandwith, 1 cm optical cuvet, and a combination of interference and edge filter in front of the photomultiplier (EMI 9558BQ) that was coupled to a transient recorder (Tektronix TDS 320). The samples were excited by saturating Xe flashes of about 15 µs duration filtered by a colored glass (CS96-4 from Corning).
3. Before measurements, the PSI complexes were diluted to approx 10 µ*M* Chl with buffer D.

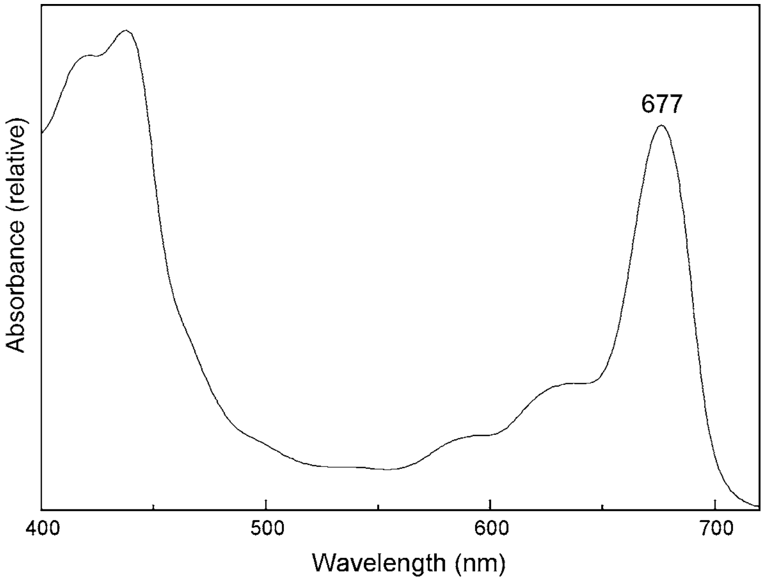

Fig. 2. Room temperature absorption spectrum of PSI core complex from CC2696 strain of *Chlamydomonas reinhardtii* with maximum absorption at 677 nm.

4. The time course of the absorbance changes was fitted to an exponential decay using an algorithm that minimizes the sum of the unweighted least squares.
5. The number of Chl molecules per RC was estimated on the basis of the ratio absorption of antenna Chls at 675 nm and ΔA_{696} owing to oxidation of P700 after a saturating flash. Assuming a molar extinction coefficient of 60 mM^{-1} cm^{-1} for a Chl *a* molecules ***(6)*** and a differential molar extinction coefficient of 64 mM^{-1} cm^{-1} for P700/P700^{+} *(7)*, we have calculated 85–90 Chls/RC for the purified PSI.
6. A femto second transient absorption spectrum (**Fig. 4**) of the PSI complex purified by ion exchange column chromatography shows a high degree of similarity in terms of excitation energy transfer within the PSI core, to what was observed previously with cyanobacterial PSI.

3.4.2. SDS-Polyacrylamide Gel Electrophoresis

1. The polypeptide composition of the core complexes was analyzed by denaturing SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of 6 *M* urea using a 10–20% linear gradient ployacrylamide gel as described by Chua *(8)*.
2. The samples were solubilized in 1% SDS sample buffer by incubation for 40 min at room temperature.
3. The gel was run for 5–6 h at 4°C with a constant voltage of 60 V. The gels were stained with coomassied Brilliant Blue R-250. For calibration, prestained marker proteins (Bio-Rad) were used.

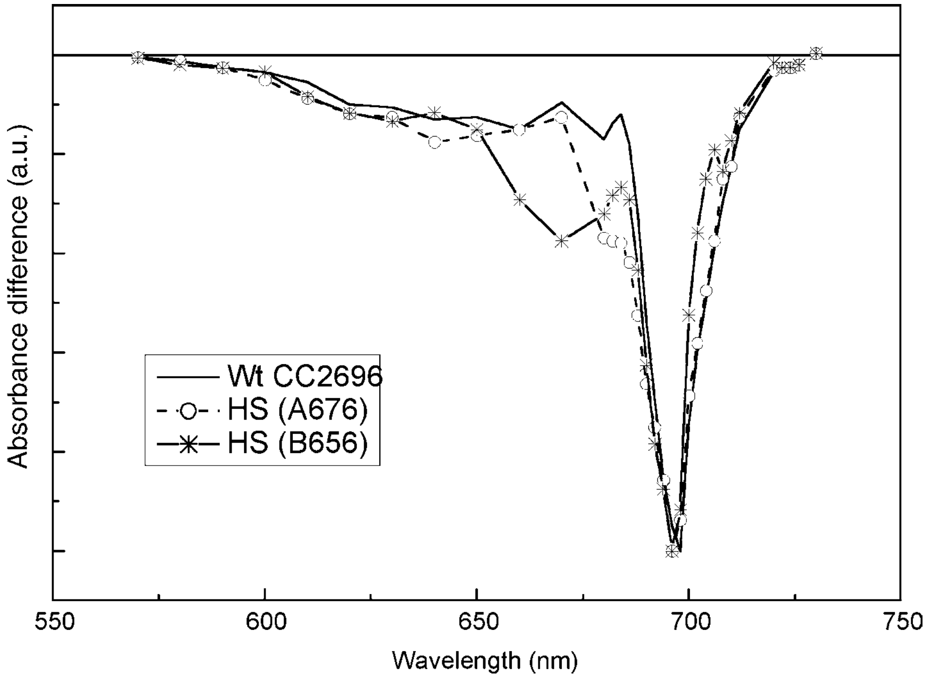

Fig. 3. Flash induced absorbance spectra of P700$^+$. /P700 measured at room temperature. The solid line without symbols (– –) represents the spectrum of the wild type (wt), and the lines with symbols represent the spectra of the mutated PSI complexes as indicated in the insets. The samples were excited by saturating flashes about 15 µs in length from a Xe flash lamb. The spectra are normalized to the same bleached area between 650 and 720 nm. This new rapid purification procedure yielded pure PSI preparations with a Chl/P700 ratio of approx 85–95 and showing typical absorption difference spectra with a maximum bleaching occurring at 696 nm.

4. Notes

1. The present method for the purification of PSI from *Chlamydomonas reinhardtii* is aimed to replace the conventional, time consuming, sucrose density gradient in order to avoid the structural and functional impairment of PSI complex. Isolation of intact PSI core complex depends upon the speed of the procedure and the mildness of the extraction and purification. The present procedure requires only 12 h to purify the active PSI.
2. Activity of photosynthetic reaction center protein is maintained by the choice of pH and the presence of glycerol and of the mild detergent dodecyl maltoside. We use Hepes buffer with pH 7.2 instead of tricine pH 7.5 (used in earlier methods), glycerol instead of sucrose or sorbitol (sucrose found to degrade the core proteins) and mild detergent β-D-maltoside. A 10–15 min treatment of thylakoid membrane

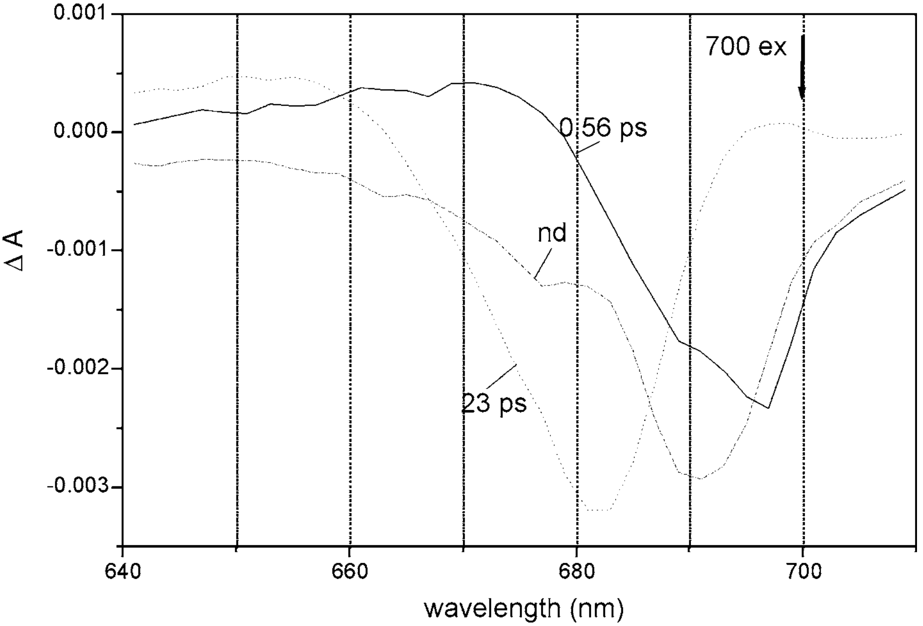

Fig. 4. Decay associated spectra of global analysis of transient absorption spectra of the PSI core antenna (purified by ion exchange column chromatography) from *Chlamydomonas reinhardtii* excited at 700 nm indicating typical 23 ps energy trapping by the reaction center.

with dodecyl maltoside extracts 80% Chl and shows no loss in electron transfer activity.

3. Use of DEAE-Toyopearl 650S anion exchange resin to purify the PSI permits us to use the low salt concentration to elute the PSI core complex from the column. $MgSO_4$ is used to elute the PSI from the column since it is most gentle salt to elute Photosynthetic reaction center proteins ***(9,10)***.
4. The utilization of protease inhibitor cocktails prevent the proteolytic cleavage of photosynthetic complexes during the breakage of cells and further steps in protein purification. Hence, it is recommended that the protease inhibitors always be added in all the buffers used for purification until the final step.
5. Changing the $MgSO_4$ for each new purification affects the elution pattern of PSI from the column. Therefore, it is recommended that the same kind (from same company) of $MgSO_4$ should always be used.
6. Step gradient 5, 8, 10, 13, 15, 18, and 25 m*M* $MgSO_4$ in buffer B can also be applied to elute the PSI from the column. Current mild purification method helped us to get 85–90 Chls/ RC for the PSI from CC 2696 *Chlamydomonas* strain. This number is higher than other PSI preparations from *C. reinhardtii* CC2696 (40–63 Chls) ***(11–13)***.

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4

Isolation of Photosystem II-Enriched Membranes and the Oxygen-Evolving Complex Subunit Proteins From Higher Plants

Yasusi Yamamoto, Shinsuke Sakuma, and Jian-Ren Shen

Summary

Methods for the isolation of highly active oxygen-evolving photosystem (PS)II membranes from higher plants and the purification of the oxygen-evolving complex (OEC) subunits are described. Membrane samples used as the material for various in vitro studies of PSII are prepared by solubilization of thylakoid membranes with the non-ionic detergent Triton X-100. The OEC subunit proteins are dissociated from the PSII-enriched membranes by alkaline treatment or salt treatment, and then purified by ion-exchange chromatography using an automated high-performance liquid chromatography system.

Key Words: Ion-exchange chromatography; membrane preparation; oxygen evolution; oxygen-evolving complex; photosystem II; protein purification.

1. Introduction

Highly active oxygen-evolving photosystem (PS)II membranes are the starting material for isolation of various membrane proteins related to the water-oxidation activity of PSII. Preparation of these membranes was first reported in the early 1980s ***(1–3)*** and promoted the structural and functional analyses of PSII. Solubilization of thylakoids with non-ionic detergents to obtain PSII-enriched grana thylakoid membrane particles is the principle technique used for preparation of membranes. The detergents Triton X-100 and digitonin were used to separate the grana-stacked from the stroma-exposed regions of the thylakoids. However, Triton X-100 was shown to be superior in yielding highly active oxygen-evolving PSII particles.

The presence of the oxygen-evolving complex (OEC) subunits in PSII was first reported from PSII-enriched oxygen-evolving samples ***(1)***. The OEC is

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composed of three extrinsic proteins, namely OEC33, 24, and 18, encoded by their respective nuclear genes *psb O, P* and *Q*, and have apparent relative molecular masses of 33, 24, and 18 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Among these proteins, OEC33 is responsible for the stabilization of catalytic Mn cluster. The exact functions of the OEC24 and 18 are not known, but it has been suggested that they are involved in the regulation of the concentration of Ca^{2+} and Cl^- necessary for the functioning of the catalytic Mn cluster. Proteins equivalent to the OEC subunit proteins of higher plants are present in PSII of green algae, and in PSII of cyanobacteria and red algae, the two small subunits are replaced by cyt c550 and a 12 kDa protein ***(4,5)***. Red algal PSII contains an additional 20 kDa extrinsic protein ***(6)***. The three OEC subunit proteins of higher plants were first isolated by isoelectric focusing (pIs of the OEC33, 24 and 18 are 5.1, 6.5, and 9.2, respectively) ***(7)***. However, these proteins are released from PSII-enriched membranes relatively easily by urea, salt, or alkaline treatment. Separation of the proteins is achieved by ion-exchange chromatography. A method to isolate large amounts of OEC33 was also reported ***(8)***. The isolated proteins have been used for various biochemical and biophysical assays of oxygen-evolving systems associated with PSII. These proteins served as models for the analyses of protein transport across the thylakoid membrane during chloroplast biogenesis ***(9, 10)***. More recently, cloned OEC subunit proteins overexpressed in *E. coli* were crystallized, allowing for structural studies of the proteins by X-ray diffraction analysis.

2. Materials

2.1. Plant Materials

The most widely used materials for isolation of the PSII-enriched membranes from higher plants are spinach and pea leaves. Spinach can be purchased in local markets. Peas are usually grown in a plant growth chamber at 20°C.

2.2. Buffers for Isolation of Actively Oxygen-Evolving PSII Membranes

Among the several PSII-enriched membranes prepared from higher plants, the BBY membranes ***(2)*** and the membranes prepared by Kuwabara and Murata (KM membranes) ***(3)*** are the most widely used.

2.2.1. BBY Membranes

1. BBY-1: 0.4 *M* NaCl, 0.2% bovine serum albumin (BSA), 20 m*M* Tricine, 2 m*M* $MgCl_2$. Add distilled water to 500 mL, and adjust pH to 8.0 with NaOH.
2. BBY-2: 0.15 *M* NaCl, 0.2% BSA, 20 m*M* Tricine, 5 m*M* $MgCl_2$. Add distilled water to 50 mL, and adjust pH to 8.0 with NaOH.

3. BBY-3: 0.4 *M* sucrose, 40 m*M* Mes, 10 m*M* NaCl, 5 m*M* $MgCl_2$. Add distilled water to 500 mL, and adjust pH to 6.5 with NaOH.
4. BBY-3T (BBY-3 + Triton X-100): 14% (w/v) Triton X-100, add BBY-3 solution to 20 mL. To dissolve Triton X-100, use a water bath or a hot-plate with a stirrer. Cool and store the solution at 4°C after preparation.
5. BBY-4: 0.4 *M* sucrose, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Mes, 30% (v/v) ethylene glycol. Add distilled water to 100 mL, and adjust pH to 6.0 with NaOH.

2.2.2. KM Membranes

1. K-1: 0.1 *M* sucrose, 0.2 *M* NaCl, 50 m*M* KH_2PO_4. Add distilled water to 3 L, and adjust pH to 6.9 with NaOH.
2. K-2: 0.3 *M* sucrose, 0.1 *M* NaCl, 50 m*M* KH_2PO_4. Add distilled water to 500 mL, and adjust pH to 6.4 with NaOH.
3. SMN: 0.4 *M* sucrose, 10 m*M* NaCl, 40 m*M* Mes. Add distilled water to 500 mL, and adjust pH to 6.5 with NaOH.
4. K-3 (the same as BBY-4): 0.4 *M* sucrose, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Mes, 30% (v/v) ethylene glycol. Add distilled water to 100 mL, and adjust pH to 6.0 with NaOH.
5. TX-100: 20% (w/v) Triton X-100. Mix the Triton into solution using a hot water bath or a hot-plate with a stirrer, and add distilled water to 50 mL. Cool and store at 4°C after preparation.

2.3. Buffers for Isolation of the OEC Subunit Proteins

2.3.1. Buffers for Isolation of OEC24 and 18

OEC24, 18 are released from PSII membranes by a 1.0 *M* NaCl-wash, and then purified by column chromatography.

1. NaCl-1: 0.4 *M* sucrose, 1.0 *M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Mes. Add distilled water to 100 mL, and adjust pH to 6.0 with NaOH.
2. A-1: 30 m*M* citric acid. Add distilled water to 500 mL, and adjust pH to 4.0 with NaOH.
3. B-1: 30 m*M* citric acid, 1.0 *M* NaCl. Add distilled water to 500 mL, and adjust pH to 4.0 with NaOH.

2.3.2. Buffers for Isolation of OEC33

OEC33 is released from NaCl-washed PSII membranes (depleted of OEC24 and OEC18) by a Tris-HCl wash and purified by column chromatography.

1. NaCl-2: 0.4 *M* sucrose, 1.0 *M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* Mes. Add distilled H_2O to 100 mL, and adjust pH to 6.0 with NaOH.
2. Tris-HCl washing: 0.4 *M* sucrose, 1.0 *M* Tris-HCl, 5 m*M* $MgCl_2$. Add distilled H_2O to 100 mL, and adjust pH to 8.5 with HCl.
3. A-2: 30 m*M* Tris-HCl. Add distilled H_2O to 500 mL, and adjust pH to 9.0 with HCl.

4. B-2: 30 m*M* Tris-HCl, 1.0 *M* NaCl. Add distilled H_2O to 500 mL, and adjust pH to 9.0 with HCl.

2.4. Other Chemicals and Materials Commonly Used for Preparation of the Membrane Samples and for Isolation of Proteins

1. Cheese cloth (for filtration of the homogenate of spinach leaves).
2. 80% Acetone (for determination of chlorophyll concentration).
4. Liquid nitrogen.
5. Dialysis tubing.

2.5. Instruments

1. Blender.
2. High-speed refrigerated centrifuge with angled rotors.
3. Brushes for resuspending membrane precipitates after centrifugation.
4. Cation exchange column Bio-Scale S (BioRad) or a similar column.
5. Anion exchange column Bio-Scale Q (BioRad) or a similar column.
6. Biologic chromatography system (BioRad) or an equivalent FPLC system.
7. Ultrafiltration cell (Millipore) connected with a high pressure nitrogen or helium gas cylinder (for dialysis and concentration of proteins).

3. Methods

3.1. Isolation of Highly Active Oxygen-Evolving PSII Membranes

3.1.1. BBY Membranes

The following steps are carried out on ice or at 4°C. Light should be avoided during the preparation if using the samples as materials for photochemical measurements or for photoinhibition studies. Green safe light is used for the preparation in the dark.

1. Rinse spinach leaves with tap water.
2. Cool leaves in ice or in a refrigerator.
3. Homogenize 100 g of spinach leaves with 300 mL BBY-1 solution in a pre-cooled blender.
4. Filter the homogenate with cheesecloth. If foaming occurs, store the homogenate in a large container in a refrigerator to diminish the foaming created during homogenization.
5. Centrifuge the filtrate at 6000*g* for 10 min.
6. Suspend the precipitate in 80 mL BBY-1 solution and centrifuge at 400*g* for 30 s. Brushes are used for resuspending precipitates (*see* **Note 1**).
7. Centrifuge the supernatant at 35,000*g* for 7 min.
8. Suspend the precipitate in 40 mL of BBY-2 solution and centrifuge at 35,000*g* for 7 min.
9. Suspend the precipitate in 7 mL of BBY-3 solution.

10. Measure the volume of the suspension with a precooled graduated cylinder (mL).
11. Determine the chlorophyll concentration in the suspension (B mg chlorophyll mL^{-1}) (*see* **Subheading 3.1.3.**).
12. Adjust the chlorophyll concentration to 2.5 mg chlorophyll/ mL^{-1} by adding [(A × B/2.5) – A] mL BBY-3 solution.
13. Add 1/4 volume of BBY-3T solution slowly to the suspension with a pipet while stirring. Avoid foaming as much as possible. The final chlorophyll concentration is 2 mg chlorophyll/mL^{-1}, and the ratio of TX-100 to chlorophyll (w/w) is 14:1. To change the ratio of TX-100 and chlorophyll, for example, to 20:1, adjust the concentration of TX-100 in the BBY-3T solution to 20%.
14. Stir slowly for 10 min.
15. Centrifuge the suspension at 35,000*g* for 15 min.
16. Suspend the precipitate in 40 mL of BBY-3 solution and centrifuge at 3000*g* for 3 min.
17. Centrifuge the supernatant at 35,000*g* for 20 min. If the resulting supernatant is green, suspend the precipitate of **step 15** again in the BBY-3 solution and centrifuge the suspension at 35,000*g* for 20 min.
18. Suspend the precipitate in BBY-4 solution.
19. Determine the concentration of chlorophyll.
20. Store the sample in small plastic sample tubes (*see* **Note 2**).
21. Freeze the samples in liquid nitrogen, and then store them at –80°C.

3.1.2. KM Membranes

1. Rinse spinach leaves with tap water.
2. Cool the leaves in ice.
3. Put leaves in a blender and homogenize with K-1 solution. Add 250 mL K-1 solution per 100 g leaves.
4. Filter the homogenate through four layers of cheese cloth.
5. Centrifuge the filtrate at 4000*g* for 5 min.
6. Suspend the precipitate with K-1 solution and centrifuge the suspension at 500*g* for 30 s (*see* **Note 1**).
7. Centrifuge the supernatant at 4000*g* for 10 min.
8. Suspend the precipitate with K-2 solution.
9. Measure the volume of the suspension with a precooled graduated cylinder (mL).
10. Determine the chlorophyll concentration (B mg chlorophyll/mL^{-1}) (**Subheading 3.1.3.**).
11. Adjust the chlorophyll concentration to 2.5 mg chlorophyll/mL^{-1} by adding [(A × B/2.5) – A]mL K-2 solution.
12. Add 20% TX-100 solution to the suspension with stirring.
13. Centrifuge at 35,000*g* for 15 min.
14. Suspend the precipitate with SMN and centrifuge at 1500*g* for 2 min. This step may be omitted.
15. If **step 14** is conducted, centrifuge the suspension again at 35,000*g* for 15 min.

16. Suspend the precipitate with K-3 solution.
17. Determine the chlorophyll concentration.
18. Transfer the samples to sample tubes (*see* **Note 2**).
19. Freeze the samples quickly in liquid nitrogen and store at –80°C.

3.1.3. Determination of Chlorophyll Concentration

1. Transfer 5 µL or 10 µL samples with a glass microsyringe to test tubes.
2. Add 2.5 mL 80% acetone and mix with a vortex mixer.
3. Centrifuge at 1300*g* for 3 to 5 min.
4. Measure absorption at 645 nm, 663 nm, and 720 nm. Calculate chlorophyll concentration using the following formulas and the reported chlorophyll absorption coefficients ***(11)***:

   Total chlorophyll (µg/mL) = (8.05 × A663 + 20.29 × A645)/(X dilution)
   Chlorophyll *a* (µg/mL) = (12.7 × A663 – 2.59 × A645)/(X dilution)
   Chlorophyll *b* (µg/mL) = (22.9 × A645 – 4.67 × A663)/(X dilution)

   To obtain A663 and A645, the absorbance equal to A720 should be subtracted from each absorbance to compensate for background absorbance. The X dilution value, for example, is 1/500 when 5 µL sample is used.

3.2. Isolation of the OEC Subunit Proteins

3.2.1. Isolation of OEC24 and OEC18

OEC24 and OEC18 are released from PSII membranes by 1.0 *M* NaCl-washing, and then purified by column chromatography.

1. Thaw PSII membranes, and then suspend them with NaCl-1 buffer to a chlorophyll concentration of 1.0 mg/mL.
2. Incubate for 30 min on ice in the dark.
3. Centrifuge the PSII membranes at 35,000*g* for 15 min.
4. Collect the supernatant containing OEC24 and OEC18 proteins. The supernatant should be colorless; if not, centrifuge it at 200,000*g* for 60 min, and then collect the supernatant again. Store the supernatant at –80°C if not used immediately.
5. Immediately before loading to a column, dilute the NaCl-washed supernatant sixfold with A-1 buffer to ensure that the NaCl concentration in the supernatant is low enough for the OEC24 and OEC18 proteins to bind to the column (*see* **Note 3**).
6. Load the NaCl-washed supernatant onto a Bio-Scale S cation-exchange column (BioRad) equilibrated with A-1 buffer.
7. Elute OEC24 and OEC18 with a linear NaCl gradient from 350 m*M* to 650 m*M* using buffers A-1 and B-1. Under this elution condition, OEC24 elutes first and then OEC18 follows (*see* **Note 4**).
8. If necessary, elute OEC24 and OEC18 as they are concentrated by ultrafiltration. The proteins are dialyzed against an appropriate buffer. Store the purified OEC24 and OEC18 at –80°C.

3.2.2. Isolation of OEC33

OEC33 is released by Tris-HCl washing of PSII membranes depleted of OEC24 and OEC18 by NaCl-washing, and then purified by column chromatography.

1. Thaw PSII membranes, and suspend them with NaCl-2 buffer to a chlorophyll concentration of 1.0 mg/mL to release OEC24 and OEC18.
2. Incubate for 30 min on ice in the dark.
3. Centrifuge the PSII membranes at 35,000*g* for 15 min.
4. Suspend PSII membrane pellet with Tris-HCl washing buffer to a chlorophyll concentration of 1.0 mg/mL; incubate for 30 min on ice in the dark.
5. Collect the supernatant, which contains OEC33, by centrifugation. The supernatant should be colorless; if not, centrifuge at 200,000*g* for 60 min, and collect the supernatant. The supernatant is stored at –80°C if not used immediately for purification by column chromatography.
6. The Tris-HCl washing supernatant is dialyzed against a large volume of A-2 buffer for three h; longer dialysis should be avoided to prevent proteolysis of OEC33.
7. Load the Tris-HCl washed supernatant onto a Bio-Scale Q anion-exchange column (BioRad) that has been equilibrated with A-2 buffer.
8. Elute OEC33 with a linear NaCl gradient from 100 m*M* to 600 m*M* using buffers A-2 and B-2. Under this elution condition, OEC33 may appear in two major fractions; collect both fractions for future use.
9. If necessary, concentrate eluted OEC33 by ultrafiltration. The protein is dialyzed against an appropriate buffer. Store the purified OEC33 at –80° before use.

4. Notes

1. Brushes for watercolor are good for suspending precipitates.
2. If necessary, black sample tubes should be used to avoid light.
3. It is important to keep the NaCl concentration in the supernatant to 1.0 *M* prior to column chromatography, because a high NaCl concentration protects OEC24 and OEC18 from digestion by proteases that may be released from PSII membranes by the NaCl-wash.
4. A small amount of OEC24 may be found in the OEC18 fraction; if this is the case, re-run the OEC18 fraction on the column. This separates the residual OEC24 from OEC18.

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5

Isolation of Functional Photosystem II Core Particles From the Cyanobacterium *Synechocystis* sp. PCC 6803

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Summary

This chapter contains the description of several methods used for the isolation of functional photosystem (PS)II core particles from wild-type (*wt*), PSI-less, and CP47 histidine-tagged cells of the cyanobacterium *Synechocystis* sp. PCC 6803. These protocols discuss the cultivation of PSI-containing and PSI-less cells, isolation of thylakoid membranes, purification of PSII core particles using a weak cation exchange or metal affinity column chromatography, and characterization of the final preparation. The described isolation procedures, which normally yield PSII particles highly active in oxygen evolution, can be easily adapted for obtaining preparations from different types of *Synechocystis* mutants with modified PSII.

Key Words: Anion-exchange chromatography; isolation protocol; metal affinity chromatography; oxygen evolution; photosystem (PS)II; *Synechocystis* 6803.

1. Introduction

Photosystem (PS)II is a multi-subunit pigment–protein complex that uses the energy of light to mediate electron transfer from water to plastoquinone in thylakoid membranes of plants, algae, and cyanobacteria ***(1,2)***. PSII is composed of more than 25 protein subunits, which include the reaction center proteins D1 and D2, the pigment-binding proteins CP43 and CP47, the α and β subunits of cytochrome b_{559}, and several Mn-stabilizing proteins of the oxygen-evolving complex (OEC). These proteins together with other polypeptides of unknown function constitute the “core” of PSII.

The unicellular photosynthetic cyanobacterium *Synechocystis* sp. strain PCC 6803 serves as an important model organism to study PSII structure and function ***(3,4)***. *Synechocystis* sp. PCC 6803 has a number of advantages over other eucaryotic and prokaryotic photosynthetic organisms. This particular strain is

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naturally transformable with high efficiency and can integrate foreign DNA into its genome by double homologous recombination, thus enabling facile introduction of mutations into the genes encoding the PSII subunits. Moreover, *Synechocystis* sp. PCC 6803 can grow photoheterotrophically, in the absence of PSII or/and of photosystem I (PSI) activities, allowing for the construction and study of PSII mutants that normally have lethal phenotype.

During the last decade, a number of protocols have been developed to isolate functional PSII complexes from *Synechocystis* ***(5–10)***. Because the PSI complex is at least five times more abundant in cyanobacterial thylakoids than PSII, it has been a challenge to separate PSII from PSI while preserving the oxygen evolution activity of the former complex. Tang and Diner ***(5)*** were the first to obtain a PSII preparation that was highly active in oxygen evolution and contained less than 4% of contaminating PSI. The procedure took at least 12 h from cell harvesting to completion and included a purification step on a weak anion-exchange column. A few years later, Bricker and colleagues ***(6)*** described an isolation of highly active PSII particles using a histidine-tagged mutant of CP47. Introduction of six histidines at the N-terminus of CP47 of *Synechocystis* had no significant effect on PSII function and allowed for the completion of the isolation procedure in less than 7 h using metal-affinity chromatography. The final preparation was highly depleted from many contaminating proteins, including PSI reaction center proteins ***(6)***. Isolation of PSII particles completely free from contaminating PSI was accomplished by using PSI-less cells ***(11)*** as a starting material, with minor adjustments of the purification protocol reported by Tang and Diner ***(7)***. The column chromatography protocols for the isolation of PSII particles from *wt*, his-tagged, and PSI-less strains of *Synechocystis* sp. PCC 6803 are described in **Subheading 3** (*see* **Note 1**).

2. Materials

1. Bead beater.
2. Glass beads, 0.1 mm in diameter.
3. Equipment for column chromatography, including a pump and a fraction collector.
4. Chromatography column packed with DEAE-Toyopearl 650S (Toso Haas) weak cation exchanger or with BD TALON (Clontech) Co^{2+} affinity resin.
5. BG-11 growth media for *Synechocystis*:
   Make stock solutions of: 3.05 g K_2HPO_4 per 100 mL H_2O; 2 g Na_2CO_3 per 100 mL H_2O; 0.6 g ferric ammonium citrate per 100 mL H_2O; trace minerals (2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.39 g $Na_2MoO_4{\cdot}2H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, 0.049 $Co(NO_3)_2{\cdot}6H_2O$ per 1 L H_2O); 0.25 *M* EDTA (pH 8.0, adjusted with NaOH) and BG-11 without iron, phosphate and carbonate (150 g $NaNO_3$, 7.5 g $MgSO_4{\cdot}7H_2O$, 3.6 g $CaCl_2{\cdot}2H_2O$, 0.6 g citric acid, 1.12 mL of NaEDTA from stock, and 100 mL of trace minerals from the stock solution per 1 L H_2O). Also make

200 mL of 1 *M* TES-NaOH buffer (pH 8.2) and 100 mL of 1 *M* filter-sterilized glucose solution, if required.

Per 1 liter of water take from stock 10 mL of BG-11 (without iron, phosphate, and carbonate), 1 mL of K_2HPO_4, 1 mL of Na_2CO_3, 1 mL of ferric ammonium citrate, and 10 mL of TES-buffer (required for growing the PSI-less strain). Sterilize the media by autoclaving on the same day. Sterile glucose (5 m*M* final concentration) can be added to the autoclaved media before starting the *Synechocystis* culture. The autoclaved BG-11 medium and sterile stock of glucose can be stored at room temperature for several months (the brownish pellet of iron salts formed in the media after autoclaving does not affect cell growth). Other stock solutions should be stored at 4°C.

6. Buffer A (thylakoid buffer): 50 m*M* MES-NaOH (pH 6.0), 10 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 25% glycerol. Store at 4°C.
7. Protease inhibitors: aminocaproic acid (dissolves in water), benzamidine hydrochloride (make 100 m*M* stock solution in ethanol, isopropanol, or dimethyl sulfoxide), and phenylmethanesulfonyl fluoride (because of a short lifetime in aqueous media, make 100 m*M* stock solution in dimethyl sulfoxide and dissolve into the buffer immediately before use). Phenylmethane-sulfonyl fluoride is toxic by inhalation, contact with skin, and if swallowed. It can be replaced with less toxic and more stable 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF).
8. Buffer B (thylakoid buffer with protease inhibitors): same as buffer A + protease inhibitors (1 m*M* of each) + 50 μg/mL DNase I (from bovine pancreas, type IV, Sigma). Prepare immediately before use.
9. Buffer C: same as buffer A, but containing 10 m*M* $CaCl_2$. Store at 4°C.
10. Detergent: 10% (w/v) stock solution of dodecyl β-D-maltoside in water. Can be stored at room temperature for at least several days.
11. Elution buffers.
    Buffer D1: same as buffer C, but containing 20 m*M* $MgSO_4$ + 0.03% w/v dodecyl β-D-maltoside.
    Buffer D2: same as buffer C + 0.03% w/v dodecyl β-D-maltoside.
    Buffer D3: same as buffer A + 0.04% w/v dodecyl β-D-maltoside.
    Buffer D4: same as buffer A + 0.04% w/v dodecyl β-D-maltoside + 50 m*M* histidine.
12. Buffer E: 50 m*M* MES-NaOH,pH 6.5, 10 m*M* NaCl, 5 m*M* $MgCl_2$, and 20 m*M* $CaCl_2$.
13. Buffer F: 40 m*M* HEPES-NaOH, pH 7.5, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* $CaCl_2$, and 0.03% (w/v) dodecyl β-D-maltoside.

3. Methods

Preparation of PSII core particles involves

1. Cultivation of a sufficient amount of cyanobacterial cells,
2. Isolation of thylakoid membranes,
3. Solubilization of thylakoid proteins and purification of PSII by column chromatography,

4. Concentration and storage of the purified material, and
5. Analysis of the quality of the obtained PSII particles.

3.1. Cell Growing

Synechocystis cells are typically grown at 28–30°C in liquid BG-11 medium ***(12)***, which should be supplemented with 5 m*M* glucose and 10 m*M* TES-NaOH buffer, pH 8.2, for the cultivation of the PSI-less mutant (*see* **Note 2**). A small amount of material (≤0.5 L) can be grown in sterile flasks on a shaker. Larger volumes require continuous pumping of filter-sterilized air through the culture. *Wt* (PSI-containing) cells are generally grown at a light intensity of 50 to 100 μmol photons/m^2s. The light intensity for growing PSI-less mutants should not exceed 5 μmol photons/m^2s. The doubling time of *wt* cells typically varies from 9 to 13 h, whereas PSI-less mutants generally double every 20–24 h.

Cells are harvested at their late logarithmic growth phase at OD_{730} approx 0.8–1.0 (*see* **Notes 3** and **4**). The protocols presented below are based on a 20-L volume of cyanobacterial culture. A 20-L carboy typically yields 17–67 nmol of PSII particles containing 0.6–2.4 mg of chlorophyll.

3.2. Isolation of Thylakoid Membranes

This subsection outlines procedures for cell disruption and isolation of thylakoid membranes. All steps should be performed at 4°C at low light or in darkness (*see* **Notes 5** and **6**).

1. Wash harvested cells by suspending them in 100 mL of buffer A. Pellet cells by centrifugation for 5 min at 6000*g*.
2. Suspend the pellet in 50 to 100 mL of buffer B (*see* **Notes 7–9**). Load the cell suspension into a pre-chilled bead beater chamber filled with 0.1 mm glass beads to give a 1:2 to 1:1 (v/v) ratio of glass beads to the cell suspension.
3. Break cells with up to 15 break cycles, where each cycle consists of 15 s of homogenization followed by 2–4 min of cooling. After breakage, separate cell homogenate from the beads by decantation; wash the beads several times with buffer B to recover additional homogenate. The final volume of cell homogenate should be approx 200 mL.
4. When isolating his-tagged PSII particles go directly to **Subsection 3.3.** Otherwise, continue from **step 5** of this subsection.
5. Remove unbroken cells and glass beads remaining in the suspension by centrifugation (5 min, 1000*g*).
6. Carefully aspirate or decant the supernatant into clean ultracentrifuge tubes (avoid taking the loose pellet). Pellet thylakoids by centrifugation for 20 min at 120,000*g* (*see* **Note 10**).
7. Suspend the thylakoids in 200 mL of buffer C and re-pellet by centrifugating for 20 min at 120,000*g* (*see* **Note 10**) to wash the material from remaining phycobilins and other soluble proteins. Discard the supernatant.

3.3. Solubilization of Thylakoids and Column Chromatography

The **Subheadings 3.3.1.**, **3.3.2.**, and **3.3.3.** contain procedures for solubilization and chromatography purification of PSII from *wt* (PSI-containing), PSI-less and his-tagged cells, respectively.

3.3.1. PSI-Containing Cells

1. Pre-equilibrate a weak anion exchange column (5.5 × 17 cm) containing DEAE-Toyopearl 650S resin (Toso Haas) with 500 mL of buffer D1.
2. Thoroughly suspend thylakoids in buffer C first with a paintbrush and then using a Potter-Elvehjem tissue grinder to a final chlorophyll concentration of 1 mg/mL (*see* **Note 11**).
3. Add dropwise a 10% stock solution of dodecyl β-D-maltoside to the thylakoid suspension to give a final detergent concentration of 1%. Proceed with the extraction for 10 min at 4°C with gentle stirring.
4. Pellet unsolubilized material by centrifugation (20 min, 40,000*g*).
5. Apply the supernatant containing the solubilized material onto the column at a flow rate of 3–4 mL/min.
6. Wash the column with 500–1000 mL of the equilibration buffer D1 at a flow rate of up to 10 mL/min under continuous monitoring of the absorption spectrum of the eluant. The wash with buffer D1 elutes in the following order; free chlorophyll and carotenoids (fractions having green and orange colors), cytochromes (fractions with a pink color characterized by an absorbance peak around 400 nm), residual phycobiliproteins (greenish-yellow color with a wide absorption peak around 620 nm), and monomeric PSI complexes (fractions with an intensive green color characterized by an absorption maximum around 679 nm). PSII, which is characterized by a red absorption maximum at about 673.5 nm, is eluted right after the monomeric PSI.
7. After the red absorption maximum of the eluant decreased from approx 679 nm to below 674.5 nm, apply a 500-mL linear gradient of 20–30 m*M* $MgSO_4$ in buffer D1. Continue to wash the column with buffer D1 containing 30 m*M* $MgSO_4$ until the red absorption maximum of the eluant does not shift above 675 nm resulting from the beginning of elution of PSI aggregates, characterized by an absorption peak around 679 nm.
8. Spool fractions containing PSII with the absorbance maxima ranging from 673.5 to 674.0 nm.

3.3.2. PSI-Less Cells

1. Pre-equilibrate a weak anion-exchange column (2.5 × 15 cm) containing DEAE-Toyopearl 650S resin (Toso Haas) with 200 mL of buffer D2.
2. Thoroughly resuspend thylakoids in buffer B first with a paintbrush, and then using a Potter-Elvehjem tissue grinder to a final chlorophyll concentration of 0.1 mg/mL (*see* **Note 12**).
3. Add dropwise a 10% stock solution of dodecyl β-D-maltoside to the thylakoid suspension to give a final detergent concentration of 0.4%. Proceed with the extraction

for 30 min at 0°C with gentle stirring. Pellet unsolubilized material by centrifugation (20 min at 40,000*g*).

4. Apply the supernatant containing the solubilized material onto the column at a flow rate of 2.5–3.0 mL/min.
5. Wash the column with 100 mL of the equilibration buffer D2 at a flow rate of 6–7 mL/min. This wash elutes free chlorophyll and carotenoids.
6. Apply a 500-mL linear gradient of 0–50 m*M* $MgSO_4$ in buffer D2 and then continue to wash the column with buffer D2, containing 50 m*M* $MgSO_4$. Collect fractions during and after the salt gradient.
7. Pool PSII fractions eluted at the end of the $MgSO_4$ gradient and during the wash with 50 m*M* $MgSO_4$ that satisfy the following criteria:
    a. The position of the peak of the Qy absorption band is in the range from 672.5 to 674.0 nm;
    b. The ratio of β-carotene absorption at approx 490 nm to chlorophyll *a* absorption at approx 673 nm is below 0.55; and
    c. No well-resolved absorption peak is observed at 420 nm (these fractions appear at the end of the PSII elution).

3.3.3. His-Tagged PSII

1. Pre-equilibrate Co^{2+} affinity column (2.5 × 10 cm) containing 25 mL of the resin (BD TALON, Clontech) with 75 mL of buffer D3.
2. Utilizing continuous gentle stirring, bring cell homogenate to a concentration of 1% dodecyl maltoside by dropwise addition of a 10% stock of the detergent. Without an additional incubation period, pellet unsolubilized material, unbroken cells, and residual glass beads by centrifugation (20 min at 40,000*g*).
3. Apply the supernatant containing the solubilized material onto the column at a flow rate of 2–3 mL/min.
4. Wash the column with 75 mL of buffer D3 at a flow rate of 4–5 mL/min.
5. Elute PSII particles from the column with buffer D4 and collect fractions. Pool the fractions with an intense green color.

3.4. Concentration and Storage of PSII Particles

PSII particles can be concentrated by using centrifugal concentrators equipped with a 30 or 50 kDa MWCO filter (e.g., Vivaspin-20, Vivascience). This approach is time consuming although it allows for the additional purification of the sample from low-molecular-weight contaminating proteins. Alternatively, PSII can be precipitated with polyethylenglycol (PEG), as described below.

1. Mix the eluted PSII particles with an equal volume of 25% PEG-8000 in buffer C containing 0.03% w/v dodecyl β-D-maltoside and incubate the mixture for 30 min on ice.
2. Harvest PSII by centrifugation (30 min, 40,000*g*).
3. Discard the supernatant and resuspend the pellet in a small volume (approx 2 mL) of buffer C containing 0.03% w/v dodecyl β-D-maltoside.

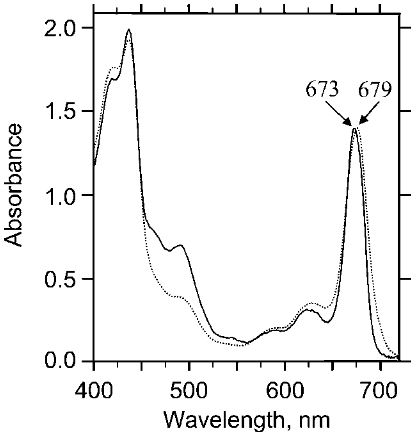

Fig. 1. Typical absorption spectra of isolated photosystem (PS)I (dashed line) and PSII (solid line) particles.

4. Put small aliquots in 0.6 mL microcentrifuge tubes, freeze in liquid nitrogen, and store in darkness at –80°C.

3.5. Quality Analysis

The quality of the purified PSII core particles can be assayed by measuring the functional activity (oxygen evolution), pigment composition, and contamination with PSI.

3.5.1. Oxygen Evolution

Oxygen evolution rates are measured using a Clark-type oxygen electrode under continuous illumination of samples at 25°C. PSII core complexes are suspended in buffer E to chlorophyll concentration of 2 to 10 µg/mL. Potassium ferricyanide (1 m*M*) and 2, 6-dichloro-*p*-benzoquinone (0.3 m*M*) are added to the suspension as electron acceptors. Upon illumination with a saturating light (1000 µmol photons/m^2s), fully active PSII particles evolve oxygen at a rate of about 2500 µmol O_2/mg chlorophyll·h.

3.5.2. Absorbance Spectra

A typical absorbance spectrum of purified PSII particles is shown in **Fig. 1**. The spectrum is characterized by large chlorophyll *a* absorption peaks at approx 436 and 673–674 nm. Absorption of pheophytin *a* is generally observed as a small peak or shoulder near 545 nm. The absorption around 460–500 nm is attributed to carotenoids (mostly β-carotene); the absorption ratio A_{490}/A_{674}

varies from 0.35 to 0.45, but may be larger if cells that were used to isolate PSII particles contained significant amount of carotenoids. The absorption ratio A_{625}/A_{674} approx 0.22 indicates that the preparation is essentially free of contaminating phycobiliproteins, which are characterized by a broad absorption band peaking at 620–623 nm.

3.5.3. HPLC Analysis

Pigments are extracted at 4°C by adding 9 volumes of cold HPLC grade acetone to 1 volume of PSII preparation (e.g., 90 µL of acetone to 10 µL of the sample). The mixture is shaken vigorously for 30 s and then centrifuged in a microcentrifuge (3 min at a maximum speed) to precipitate proteins. To avoid pheophetinization of chlorophyll, the extract is immediately injected in a chromatograph for analysis on a reverse phase C-18 HPLC column (e.g., Spherisorb S5ODS1; 250 × 4.6 mm). The column is eluted at a flow rate of 1 mL/min with an isocratic mobile phase consisting of methanol and ethyl acetate (68:32, v:v). The pigments are detected at 663 nm (chlorophyll *a* and pheophytin *a*), 450 nm (β-carotene), and 254 nm (plastoquinone-9). Extinction coefficients for chlorophyll *a*, pheophytin *a*, and β-carotene dissolved in the methanol/ethyl acetate mix (68:32, v:v) are 86.9 mM^{-1} cm^{-1} at 663 nm, 49.3 mM^{-1} cm^{-1} at 663 nm, and 135.0 mM^{-1} cm^{-1} at 450 nm, respectively ***(13)***. The extinction coefficient of plastoquinone-9 dissolved in absolute ethanol is 17.9 mM^{-1} cm^{-1} at 254 nm ***(14)***. A typical PSII preparation contains 33–40 chlorophyll *a* molecules, 10 β-carotenes, and approx 2 plastoquinones per two pheophytins.

3.5.4. Contamination With PSI

Contamination of the preparation with PSI can be assessed by measuring ascorbate-reduced minus ferricyanide-oxidized absorption difference spectrum using a double-beam spectrophotometer. PSII particles are suspended in buffer F to OD_{674} approx 1 and then placed in two different cuvets. P700 is oxidized in the first cuvet by adding ferricyanide (20 µM). A few granules of solid sodium ascorbate and phenazine methosulfate (20 nM) are added to the second cuvet and the difference absorption spectrum is recorded a few minutes later. The upper value for the concentration of P700 can be calculated using the differential absorption coefficient $\Delta A_{705-750} = 64$ mM^{-1} cm^{-1} ***(15)*** and the percentage of chlorophyll molecules from contaminating PSI can be estimated considering that each PSI complex can contain 96 chlorophylls ***(16)***.

4. Notes

1. PSII core particles can also be purified by gradient centrifugation in sucrose, or gradient centrifugation can be used as an additional purification step in combination with column chromatography ***(8–10)***. One of the major disadvantages of this

approach is that it cannot be scaled up to isolate large quantities of the material often required for spectroscopic studies. There were no reports indicating that preparations obtained by sucrose density centrifugation retain significant oxygen evolution activity.

2. To accelerate cell growth, glucose (5 m*M* final concentration) is often added to wt *Synechocystis* cultures as well.
3. The suspension of wt *Synechocystis* cells with OD_{730} = 1 (measured in a 1-cm cuvet using a Shimadzu UV-150 spectrophotometer) contains 4–5 µg chlorophyll/mL (0.8–1.0 µg chlorophyll/mL for PSI-less cells). Growing cultures to higher optical density may result in less efficient cell breakage.
4. Cyanobacterial cells can be harvested by 5-min centrifugation at 3000*g* using a conventional centrifuge. Considering the large volume of starting material, it is desirable to use rotors accommodating large-volume buckets that allow concentration of 4 to 6 L of cell suspension in one run. In this case, the harvesting of 20-L culture can be completed in 1.5–2.0 h. Another option is to use a continuous flow rotor /centrifuge, or a special filtration apparatus for bacterial cells (e.g., Pellicon tangential ultrafiltration device equipped with 0.4 µm filter, Millipore).
5. Because PSII complexes are easily and irreversibly damaged by illumination, exposure of the material to light should be reduced to a minimum. It is best to perform the isolation in a cold room under a dim green light or in darkness, whenever possible.
6. Freezing of cells or isolated thylakoids in liquid nitrogen for their subsequent storage at low temperature in between the isolation steps results in partial loss of oxygen-evolving activity of the final preparation ***(5)***. Therefore, only the fresh material should be used for the isolation of PSII. If the isolation procedure cannot be completed in 1 d, minimal loss of oxygen evolution occurs if solubilized PSII particles are left overnight on the DEAE column (the column with PSII should be preliminarily washed with 0.03–0.04% dodecyl maltoside dissolved in the corresponding washing buffer and then stored in complete darkness at 4°C).
7. The pelleted material (cells, thylakoids, or purified PSII particles) is easily suspended with a small paintbrush soaked in ice-cold wash buffer.
8. The optimal pH for the isolation of oxygen evolving PSII particles is about 6.0–6.4. Higher pH (7.2–7.4) cause partial loss of oxygen evolution. However, the chlorophyll to pheophytin ratio of the final preparation tends to be higher, if the isolation is performed at slightly basic pH.
9. The volume of cell suspension and the amount of added glass beads depend on the type of available bead beater chamber. Biospec Products (Bartlesville, OK) offers several models of bead beaters suitable for different volumes of cell suspension. Variations in the concentration of cell suspension and the amount of glass beads within certain limits do not affect the efficiency of cell breakage.
10. Because most of thylakoids can be pelleted by 30 min centrifugation at a lower centrifugal force (approx 40,000*g*), the isolation protocol can be adapted to use an ordinary centrifuge (e.g., Sorvall RC-5 centrifuge equipped with SS-34 rotor) rather than an ultracentrifuge.

11. Chlorophyll concentration is determined by pigment extraction with acetone or methanol (100% methanol is good for chlorophyll extraction from unbroken cells). Proteins are precipitated from the extract by 3-min centrifugation in a bench microcentrifuge at maximum speed. The specific extinction coefficients for chlorophyll dissolved in 100% methanol (λmax = 665.2 nm) and in 80% acetone (λmax = 663.6 nm) are 80.0 mL·mg^{-1} cm^{-1} and 86.0 mL·mg^{-1} cm^{-1}, respectively ***(17)***.
12. PSI-less cells have nearly fivefold reduced chlorophyll content as compared to *wt Synechocystis*. Therefore, initial solubilization of thylakoid membranes in dodecyl maltoside is performed at a lower chlorophyll concentration. The best results were obtained when final concentration of the detergent was reduced to 0.4%, but the solubilization continued for a longer time (30 min) comparing to *wt* cells.

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6

Isolation of Photosystem I Reaction Center Preparation From Spinach

Tetsuo Hiyama

Summary

This chapter deals with the preparation of photochemically active reaction center particles (Type III) that basically contain only two subunits, PsaA and PsaB. The preparation is obtained by further treating the Type II preparation described in the preceding chapter with a chaotropic agent, sodium iodide, followed by ultrafiltration.

Key Words: Photosystem (PS)I; reaction center preparation.

1. Introduction

This chapter deals with the preparation of the Type III reaction center particles as described in Chapter 2. The Type III contains two subunits, PsaA and PsaB (**Note 1**). The preparation is obtained by further treating the Type II preparation with either a chaotropic agent, sodium iodide (NaI) or a strong detergent (lithium dodecyl sulfate) followed either by a gel-permeation chromatography or by ultrafiltration *(1)*. Described here is an NaI-ultrafiltration method that has been employed successfully in my laboratory for years (**Note 2**).

2. Materials

1. Type II preparation described in Chapter 2.
2. NaI medium: 2 *M* NaI, 0.1% Triton X-100 and 10 m*M* Tris-HCl, pH 8.8 (*see* **Note 3**).
3. Dilution medium: 0.1% Triton X-100 and 10 m*M* Tris-HCl, pH 8.8.
4. Purified nitrogen gas (99.9998% or better).
5. Ultrafiltration apparatus with a stirring device (Ultrafiltering Unit, Advantec, Tokyo; Stir Cell, Model 8200, Millipore) and pressurized gas and ultrafilter disc (UK-50, Advantec, Tokyo; Biomax PBQK Ultrafiltration Discs or Ultracel PLTK Ultrafiltration Discs, Millipore).

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6. Nitrogen gas (regular grade) tank with a regulator and fittings for the above apparatus.
7. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) apparatus with a gradient gel maker as described in Chapter 2.

3. Methods

The starting material is the Type II preparation described in Chapter 2. It is important to perform the experiments in a cold room and keep it anaerobic to keep photochemical activities intact.

3.1. Preparation

1. Deoxygenate the NaI and dilution media by flushing with purified nitrogen gas and chill prior to use (*see* **Note 4**).
2. Suspend the Type II preparation in the NaI medium (100 mL, final protein concentration, 0.1 mg/mL) and place in a nitrogen-filled ultrafiltration apparatus fitted with the ultrafilter disc.
3. Apply pressurized nitrogen gas (2.5 kg/cm^2) immediately with stirring.
4. Set the filtrate aside for subunit preparations described in the following chapter.
5. Dilute the concentrate left on the filter (approx 10 mL) with 100 mL of the dilution medium, and filtrate again.
6. Repeat this procedure three times.
7. Supplement the final concentrate with 20% sucrose immediately and keep at –80°C for storage.

3.2. Assays

3.2.1. SDS-PAGE

Perform as described in a previous chapter. A typical SDS-PAGE pattern is shown in **Fig. 1**.

3.2.2. P700 Determination

This method is optional because it requires specialized equipment (*see* **Note 6** of Chapter 2 and **ref. *1***). It should be noted that the photochemical activity is less stable in this type of preparation than Type II; chemical difference spectrum is all but impossible. Flash-induced absorbance changes can be measured by using a custom-made high-speed flash spectrophotometer *(1)*.

4. Notes

1. Recently, another small subunit protein, PsaY/PsbW, was reported to be present ubiquitously in Type III *(2)*.
2. NaI-method has been used reproducibly in our laboratory for years for obtaining photochemically active preparations. Other methods are described in **ref. *2*** in detail. A photochemically inactive (but useful for in vitro reconstruction of P700 by sup-

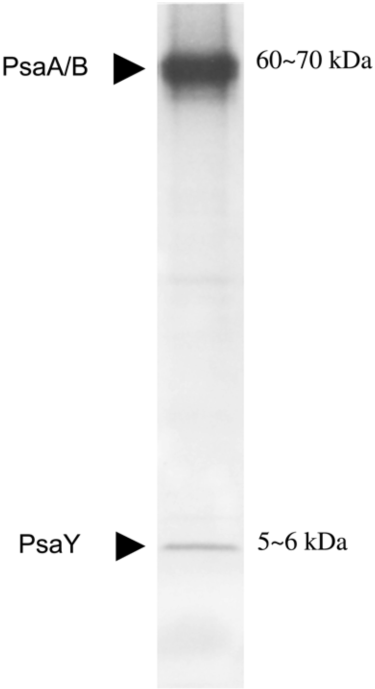

Fig. 1. SDS-PAGE of photosystem I reaction center preparation (Type III) from spinach. Electrophoresis was performed as described in the text.

plementing chlorophyll *a* epimer (*a*′) was prepared by using a strong detergent (lithium dodecyl sulfate) followed by gel-permeation chromatography at room temperature ***(3)***.

3. The pH values of the media and buffers described are all measured and adjusted at room temperatures.
4. Deoxygenation is not necessary for a photochemically inactive preparation.

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7

Isolation of Photosystem II Reaction Center Complexes From Plants

Michael Seibert, Inmaculada Yruela, and Rafael Picorel

Summary

Methods to isolate and purify 6- and 5-chlorophyll (Chl) D1/D2/Cyt b_{559} photosystem (PS) II reaction center complexes from plants are presented, and the advantages and disadvantages of each procedure are discussed. One of the simpler 6-Chl procedures and a procedure for isolating 5-Chl complexes are described in detail. Furthermore, a rapid procedure that produces relatively large amounts of less pure 6-Chl material is also described. Criteria to assess the purity of photosystem II (PSII) reaction center preparations are presented, and problems associated with each of the isolation procedures are discussed.

Key Words: Intrinsic protein; isolation; pigment–protein complex; photosystem II; reaction center.

1. Introduction

The reaction center (RC) complex of photosystem II (PSII) was first isolated by Nanba and Satoh ***(1)*** 19 yr after the bacterial reaction center was purified. Both are integral membrane protein complexes, and the latter was the first such complex for which an X-ray crystal structure was obtained ***(2)***. The isolation of the PSII RC, containing the D1 and D2 polypeptides and Cyt b_{559}, started a long series of events that have led to many advances in understanding functional aspects of PSII and its structure at the molecular level ***(3,4)***. Although the PSII RC complex itself has not been crystallized so that a detailed crystal structure could be obtained, X-ray crystal structures of PSII core complexes that contain the RC have been obtained ***(5–6)***. Nevertheless, this chapter will illustrate some common procedures used to isolate the PSII RC from plants and the pitfalls that researchers might face in the process. The procedures described have been optimized for spinach, pea, and sugar beet.

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2. Materials

1. Plant leaves such as from spinach (*see* **Note 1**), pea, and sugar beet, but modifications of the procedures have worked with wheat and *Spirodella.*
2. Warring blender and cheesecloth.
3. Cold room or cold box.
4. Preparative centrifuge, centrifuge rotors capable of generating forces of up to 33,000g, and centrifuge tubes (*see* **Note 2**).
5. Spectrophotometer for determining chlorophyll concentrations and absorption spectra.
6. Detergent (Triton X-100 and *n*-dodecyl-β-D-maltoside [β-DM]), buffers, sucrose, and common salts.
7. Anion-exchange resin, TSK-GEL Toyopearl DEAE 650(S) (*see* **Note 3**).
8. Chromatographic columns (2.5 × 9 cm, 1.6 × 10 cm, and 1 × 10 cm).
9. Pumps and fraction collector or FPLC.
10. Linear gradient maker.
11. Dialysis tubing.
12. Centricon or Centriprep centrifugal concentrator tubes.
13. Buffer K-1: 50 m*M* Na/K phosphate (pH 7.4), 100 m*M* sucrose, and 200 m*M* NaCl.
14. Buffer K-2: 50 m*M* Na/K phosphate (pH 6.9), 300 m*M* sucrose, and 50 m*M* NaCl.
15. Buffer K-3: 40 m*M* Na/K phosphate (pH 6.9).
16. Buffer K-4: 20 m*M* Mes-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.
17. Buffer A: 50 m*M* Tris-HCl (pH 7.2) and 30 m*M* NaCl.
18. Buffer B: 50 m*M* Tris-HCl (pH 7.2), 30 m*M* NaCl, and 0.05% (w/v) Triton X-100.
19. Buffer C: 50 m*M* Tris-HCl (pH 7.2) and 0.05% (w/v) β-DM.
20. Buffer D: 50 m*M* MES (pH 6.5) and 30 m*M* NaCl.
21. Buffer E: 50 m*M* MES (pH 6.5), 30 m*M* NaCl, and 1% (w/v) Triton X-100.
22. Buffer F: 50 m*M* MES (pH 6.5) and 0.05% (w/v) Triton X-100.
23. Buffer G: 50 m*M* MES (pH 6.5) and 0.05% (w/v) β-DM.
24. Buffer H: 50 m*M* Na_2HPO_4 (pH 6.5), 50 m*M* NaCl, 0.2% (w/v) Triton X-100 and 0.06% (w/v) β-DM.
25. Buffer I: 50 m*M* MES (pH 6.5), 5 m*M* imidazol, and 0.1% (w/v) β-DM.

3. Methods

The methods outlined below describe how to:

1. Isolate PSII membrane fragments, required for isolating and purifying PSII RC material,
2. Isolate standard 6-chlorophyll (Chl) per center (2 pheophytins) PSII RC preparations using anionic-exchange chromatography,
3. Isolate 5-Chl per RC preparations using immobilized metal affinity chromatography (IMAC), and
4. Determine the purity of the preparations.

In addition to step 2 above, a procedure will also be described to rapidly isolate large amounts of 6-Chl-type preparations when the purity of the RCs is not a major issue.

3.1. Isolation of PSII RC Complexes

Isolated PSII-enriched membrane fragments are the starting material for the further isolation and purification of PSII RC material. In 1981 and 1982, three groups reported the use of different detergent-solubilization procedures to isolate active PSII membranes (i.e., membranes that still exhibited water-splitting function) from broken thylakoid preparations ***(7–9)***. Dunahay et al. ***(10)*** reported a detailed comparison of the purity of the different types of PSII membrane fragments. It is important for the purpose of RC-isolation protocols that the PSII membranes are not contaminated with photosystem I (PSI) because PSI complexes, if present may elute with the PSII RC complex. See Chapter 4 for more information about the preparation of PSII membrane fragments. The following protocol is used in our laboratories, and it is a slight modification of the so-called KM procedure ***(8,10)***. However, the BBY procedure ***(7,10)*** can also be used.

3.1.1. Procedure for Isolating PSII Membrane Fragments

1. The following steps should be performed at 4°C in dim room light (**steps 2** and **3**) and in the dark (**steps 4–11**). The buffers referred to in this section are described in Table 1.
2. In a Warring blender, grind 1 Kg of de-ribbed spinach in ice-cold K-1 buffer (1:2 w/v). Repeat four to five times for 10 s each time with about 30 s in between each grind until the green suspension does not contain leave debris larger than a few mm. Filter the suspension through 16 layers of cheesecloth.
3. Centrifuge the filtrate at 500*g* (e.g., 2000 rpm in a Beckman JLA-10.500 rotor) for 1 min to remove any cell debris that passes through the cheesecloth.
4. Pour the supernatant into appropriate sized centrifuge tubes and pellet at 12,300*g* (e.g., 10,000 rpm in a Beckman JLA-10.500 rotor) for 25 min.
5. Discard the supernatant (it contains free Chl, cell cytoplasmic material, proteases, etc.), resuspend the pellet in a smaller volume fresh K-1 buffer, pour into centrifuge tubes, and pellet at 12,300*g* (e.g., 10,000 rpm in a Beckman JLA-10.500 rotor) for 25 min.
6. Resuspend the pellet in K-2 buffer with a homogenizer and run a Chl analysis. Adjust the suspension to between 3 and 4 mg Chl/mL. The Chl a/b ratio should be around 3.0 if the procedure is successful at this point.
7. Add 20% (w/v) Triton X-100 to the suspension with gentle stirring to a final ratio of 25:1 (w/w) Triton/Chl (*see* **Note 1**). Incubate with gentle stirring for 7 min in the cold. The suspension should turn a deeper shade of green.
8. Pour the suspension into clean centrifuge tubes and pellet at 32,900*g* (e.g., 20,000 rpm in a Beckman JA-25.50 rotor) for 15 min.

9. Discard the supernatant, resuspend the pellet in K-3 buffer with a homogenizer, and pellet at 737*g* (e.g., 3000 rpm in a Beckman JA-25.50 rotor) for 1.5 min to remove starch.
10. Pour the supernatant into clean centrifuge tube and pellet at 32,900*g* (e.g., 20,000 rpm in a Beckman JA-25.50 rotor) for 15 min.
11. Resuspend the pellet in K-4 and run a Chl analysis. A Chl a/b ratio of 1.8–2.2 indicates a successful preparation at this point. The membranes can be stored under liquid N_2 for up to 1 yr.

3.1.2. Procedures for Isolating and Purifying 6-Chl PSII RC Complex

This procedure is based on the original by Nanba and Satoh ***(1)***, which produced highly labile material ***(11,12)***. Stabilization of the material was accomplished by exchanging the non-ionic detergent β-DM (or lauryl maltoside) for the non-ionic detergent Triton X-100 ***(11,13)***. However, even after purification and stabilization, if the material is to be left unfrozen in room light for more than a short period of time, the researcher should work at 4°C with an oxygen-scrubbing system added to the material ***(13)***.

3.1.2.1. STANDARD PROCEDURE FOR PREPARING HIGHLY PURIFIED RCS (0.05% TRITON X-100)

This protocol produces a highly purified PSII RC preparation, but may take several days to complete (around 72 h with the starting material suggested below; *see* **Note 4**), depending on how much material is to be isolated.

1. The following steps should be performed at 4°C in the dark.
2. Unfreeze 62 mg Chl of PSII-enriched membranes and pellet at 32,900*g* (e.g., 20,000 rpm in a Beckman JA-25.50 rotor) for 15 min.
3. Resuspend the pellet in 48 mL (final volume) of buffer A.
4. Add 12 mL 20% (w/v) Triton X-100 (60 mL total final volume at 1 mg Chl/mL and 4% w/v Triton) slowly with gentle stirring and incubate for 1 h also with gentle stirring.
5. Centrifuge at 32,900*g* for 1 h and keep the supernatant.
6. Load the supernatant onto a pre-packed (at 4°C) TSK-GEL Toyopearl DEAE 650(S) anion exchange column (1.6 × 10 cm) pre-equilibrated with buffer B.
7. Wash the column overnight with the same buffer at a flow rate of 2.5 mL/min until the eluate exhibits a 417:435 nm peak ratio of about 1.16 (this ratio is approximately the ratio of the final RCs as purified). This ratio is a better measure of final purity than the absorbance of the eluate at 670 nm, which depends on the column flow rate. The wash step takes about 30 h. Higher amounts of starting material will take longer periods of time to wash.
8. Exchange β-DM for Triton X-100 by washing the column with the same buffer except with 0.1% (w/v) β-DM substituted for the Triton. Continue to wash until

the absorption band at 280 nm (residual Triton) has an absorbance lower than 0.1 (1-cm path).

9. Apply 140 mL of a 30–200 m*M* NaCl linear gradient (in the same buffer and detergent) at a flow rate of 1 mL/min. Collect 3 mL fractions of the eluate and save. The green band containing the RC complex elutes at 90–120 m*M* salt. Note that two peaks may come off the column, but the second is much smaller than the first. If so use the peak that comes off first because it is purer (*see* **Note 5**).
10. Take the absorption spectrum of each fraction, combine those with a red-peak maxima at 675 nm or higher, and desalt by dialyzing against buffer C for 1 h with one buffer exchange. Desalting is required if the samples are to be used for low-temperature spectroscopy, since high salt will interfere with the formation of a clear glass.
11. If desired, the material can be concentrated using Centriprep YM-50 centrifugal filter devices (50,000 MW cut-off; Amicon Bioseparations, Bedford, MA) or Centricon-50 (50,000 MW cut-off, Amicon, Beverely, MA) concentrators, if small volumes are to be concentrated. When the desired concentration is achieved, freeze the sample in liquid N_2 and store under liquid N_2. The samples can also be stored at –80°C in a freezer if they are to be used within a short period of time or on dry ice if they are to be shipped.

3.1.2.2. MODIFIED PROCEDURE FOR FASTER PREPARATION OF RCS WITH HIGH YIELD BUT LOWER PURITY (1% TRITON X-100)

This procedure can be used if large amounts of material are needed, and contamination from nonpigmented proteins is not a significant issue. The advantage of this protocol is that the isolation procedure takes less time (**steps 2–12** in **Subheading 3.1.2.2.** take 20 h with the suggested starting material) (*see* **Note 6**).

1. The following steps should be performed at 4°C in the dark.
2. Unfreeze 62 mg Chl of PSII-enriched membranes.
3. Centrifuge the suspension at 32,900*g* (e.g., 20,000 rpm in a Beckman JA-25.50 rotor) for 15 min. Resuspend the pellet in buffer D (*see* **Note 7**) to a final volume of 48 mL.
4. Add 12 mL 20% (w/v) Triton X-100 (60 mL total final volume at 1 mg Chl/mL and 4% w/v Triton) slowly with gentle stirring and incubate for 2 h also with gentle stirring.
5. Pellet at 32,900*g* for 1 h.
6. Load the supernatant onto a pre-packed TSK-GEL Fractogel DEAE 650(S) column (2.5 × 9 cm) that had been pre-equilibrated with buffer E.
7. Wash the column with the same buffer for 2 h at a flow rate of 2 mL/min, and then apply 170 mL of a 60–400 m*M* NaCl linear gradient in the same buffer but with 0.05% (w/v) Triton X-100 at a flow rate of 1 mL/min.
8. Pool the 3 mL fractions with a distinct Q_x Pheo band observed at around 543 nm.

9. Dilute the pooled eluate eightfold with buffer F, and then load it onto a smaller TSK-GEL Fractogel DEAE 650(S) column (1.6 × 2.5 cm) pre-equilibrated with buffer E (*see* **Note 8**).
10. Wash the column overnight with the same buffer at a flow rate of 1 mL/min.
11. When the eluate shows a 417:435 nm peak ratio of about 1.16 (the wash step takes about 14 h), exchange β-DM for Triton X-100 by further washing with 45 mL of the same buffer, but with 0.1% (w/v) β-DM substituted for the Triton.
12. Elute the RC complex with 140 mL of a 60–350 m*M* NaCl linear gradient in the same buffer with 0.1% β-DM at a flow rate of 1 mL/min (the green band elutes at about 170 m*M* salt).
13. Take the spectra of each 3 mL fraction, combine those with a red-peak maximum at 675 nm or higher and desalt by dialyzing against buffer G for 1 h with one buffer exchange (*see* **Note 9**).
14. If desired, the material can be concentrated using Centriprep YM-50 centrifugal filter devices (50,000 MW cut-off; Amicon Bioseparations, Bedford, MA). When the desired concentration is achieved, freeze the sample in liquid N_2 and store under liquid N_2. The samples can also be stored at –80°C in a freezer if they are to be used immediately or on dry ice if they are to be shipped.

3.1.3. Procedure for Isolating and Purifying 5-Chl PSII RC Complex

This procedure was developed by Vacha et al. ***(14)***. This preparation is directly eluted with β-DM, but may be less stable than the 6-Chl preparations because one of the proximal Chls is missing. Note that an oxygen-scrubbing system can be added to this preparation to improve stability. The entire isolation procedure takes 30 h with the amount of starting material suggested below.

1. The following steps should be performed at 4°C in the dark.
2. Unfreeze 31 mg Chl of PSII-enriched membranes and treat with 2 *M* $CaCl_2$ for 10 min with gentle stirring.
3. Centrifuge the suspension at 32,900*g* (e.g., 20,000 rpm in a Beckman JA-25.50 rotor) for 15 min and resuspend the pellet in 50 m*M* MES (pH 6.5) at a Chl concentration of 2 mg Chl/mL.
4. Solubilize by adding 1 vol. of 30% (w/v) Triton X-100 to 3 vol. of treated membranes, and incubate for 2 h with gentle stirring.
5. Centrifuge at 40,000*g* (e.g., 22,000 rpm in a Beckman JA-25.50 rotor) for 30 min to remove any non-solubilized material.
6. Load the supernatant onto a Cu (II) affinity column (1 × 10 cm) at a flow rate of 4 mL/min. The column material is prepared by washing Fast-Flow Chelating Sepharose (cat. no. 17-0575-01; Amersham [Pharmacia], Uppsala, Sweden) with distilled water, and then degassing the material under vacuum to remove O_2 bubbles that could interfere with the chromatography. At this point the material is packed into the 1 × 10 cm column. The column is prepared at room temperature, washed extensively with distilled water and then with 10 mL of 0.1 *M* $CuSO_4$.

The excess Cu is removed by washing the column with 100 mL of distilled water, and finally the column is equilibrated with 50 mL of buffer H at 4°C. The use of both detergents seems to improve the stability of the preparation.

7. Wash the loaded column at a flow rate of 2.5 mL/min with buffer H until the 417:435 nm peak ratio of the eluate is about 1.16.
8. Elute the column with buffer I at a flow rate of 1 mL/min, and collect 3.0 mL fractions.
9. Take the absorption spectra of each fraction and pool those with a red-peak maximum at 677 nm or higher.
10. If desired, the material can be concentrated using Centriprep YM-50 centrifugal filter devices (50,000 MW cut-off; Amicon Bioseparations, Bedford, MA) or Centricon-50 (50,000 MW cut-off, Amicon, Beverely, MA) concentrators, if small volumes are to be concentrated. When the desired concentration is achieved, freeze the sample in liquid N_2 and store under liquid N_2. The samples can also be stored at –80°C in a freezer if they are to be used within a short period of time or on dry ice if they are to be shipped.

3.2 Purity of the Preparations

3.2.1. UV-VIS Absorption Spectra

Room-temperature absorption spectra of 6-Chl RCs, obtained using either low (0.05% w/v) or high (1% w/v) Triton X-100 concentration in the column-washing buffers, exhibit a red-band maximum at 675.0–675.5 nm resulting from the Q_y transition of Chl and Pheo, a 435 nm band resulting from Chl, and a 417 nm band mainly resulting from Pheo (but also to the Soret bands of Chl and Cyt b_{559}). The distinct band at 543 nm corresponds to the Pheo Q_x transition, and the bands between 450 and 525 nm to β-carotene. The spectrum of a 5-Chl RC preparation is very similar to that of the 6-Chl RC material with the exception that the red-band peak is at 677 nm, and the absorption at around 670 nm is smaller by comparison.

3.2.2. Pigment Quantitation

Determination of the Chl *a*, Pheo *a*, and β-carotene contents in all three PSII RC preparations can be done by spectral analysis of the pigments extracted in 80% (v/v) acetone following the method described in ***(15)***. The pigments (*see* **Note 10**) are extracted at room temperature under dim light, sonicated for 1 min, and centrifuged for 3 min in a microfuge to remove insoluble material. Complete pigment extraction is assured by repeating the extraction procedure twice and checking that no pigments (except Cyt b_{559}) remain in the pellet. Note that the extract needs to have an optical density of between 0.3 and 0.6 absorption units in the Q_y absorption region, to accurately calculate the pigment concentrations below, from absorption values found in the lower absorption regions. The following equations are used to determine the pigment concentrations where c and A are given in μM and cm^{-1}, respectively.

$$c_{\mathrm{Chl}} = 11.577\ A_{663} - 76.994\ A_{535-551} + 0.624\ A_{480}$$
$$c_{\mathrm{Pheo}} = 0.020\ A_{663} + 132.505\ A_{535-551} - 1.150\ A_{480}$$
$$c_{\mathrm{Car}} = -\ 0.146\ A_{663} - 4.054\ A_{535-551} + 8.311\ A_{480}$$

3.2.3. Polypeptide Content Determination by SDS-PAGE

Coomassie Brilliant Blue staining of a gel (SDS-PAGE containing 12.5% acrylamide) demonstrates that all three types of RCs contain the same polypeptides. The heaviest staining bands are at around 33 and 30 kDa, which correspond to D2 and D1, respectively, depending on the presence of urea in the gel. A weakly staining band at 55–60 kDa corresponds to a cross-linked D1-D2 heterodimer, and the low molecular weight band (9.5 kDa) corresponds to the α-subunit of the Cyt b_{559}.

4. Notes

1. If market spinach is used, we have found that material purchased in Denver between the months of April and October produce adequate RC material. If spinach is purchased during the winter months, it can be difficult to (a) get membranes unless the Triton/Chl ratio is lowered or (b) separate RC and antenna complexes during RC isolation.
2. The centrifugation times may vary with conditions. The times given are the longest times that we have found necessary.
3. TSK-GEL Toyopearl DEAE 650(S) can be stored at room temperature or at 4°C; however, it should not be frozen. The user is cautioned that the material is an eye and skin irritant, and is extremely flammable if dried. Refer to the instructions for preparing the resin prior to packing a column and for regenerating the material after use.
4. Increasing the column flow rate somewhat can shorten the time-consuming, column-washing steps (although more buffer will be used), if precautions are taken to ensure that everything on-line (column, resin, fraction collector, tubing, and pumps, for example) has the capability to maintain the higher flow rate. Note that the viscosity of a solution containing detergent is dependent on the temperature, and thus, at 4°C the viscosity is much higher than that at room temperature where all the chromatography tools and method specifications are normally checked.
5. Higher levels of the D1-D2 heterodimer band at 55–60 kDa in the SDS-PAGE indicate a damaged RC preparation. The ideal situation is a nondetectable level of the heterodimer.
6. The RC preparations obtained with high Triton X-100 concentration (1% w/v) tend to have less β-carotene content.

7. The use of buffers at pH 6.5 (compared to higher pHs) results in a more transparent (less turbid) final preparation of RC.
8. When Taurine at a concentration of 1.5% (w/v) is added to the column-washing buffers, the RC-6 Chl preparation obtained with high Triton X-100 concentration (1% w/v) has less nonpigmented protein contaminant, but the yield of the isolated material is lower.
9. The 6-Chl RC preparation obtained with high Triton X-100 concentration (1% w/v) in the column-washing buffer can be further purified, if necessary, using a sucrose density gradient. To make the gradient, a solution of 50 m*M* MES, pH 6.5, 500 m*M* sucrose, and 0.1% (w/v) β-DM is placed in 10 mL centrifuge tubes. Tubes containing this solution are frozen at –20°C and allowed to slowly thaw at 4°C. This procedure will form a sucrose gradient. The tubes are then loaded with contaminated RC material, placed in a swinging bucket rotor (e.g., a Bechman Ti SW41 rotor), and centrifuged overnight at 35,000 rpm at 4°C. A major green band appears at the middle of the gradient, and this is the pure RC material. We emphasize that the contaminating material is nonpigmented since the pigment content of the preparation is 6 Chls/2 Pheos before and after the sucrose density step. Any green pigmentation in the pellet appears to be aggregated RC.
10. Recent studies ***(16,17)*** reveal differences in the spectroscopy of the pigments associated with P680 in PSII core vs PSII RC preparations resulting perhaps from structural changes in the reaction center accompanied by the loss of Q_A. This observation provides new opportunities for the use of the preparations described in this paper for characterizing the effects of structure on the spectroscopy of the reaction center pigments.

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8

Isolation of Photosystem I Reaction Center Subunit Polypeptides From Spinach

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Summary

Methods to prepare photosystem (PS)I reaction center (RC) subunit polypeptides are described. For PsaC, PsaD, PsaE, and PsaY, the filtrate resulting from the ultrafiltration in Chapter 6 is used for preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). PsaA and PsaB, Type II PSI particles, prepared as described in the preceding chapter, are treated with sodium dodecyl sulfate, then applied on preparative SDS-PAGE. After electrophoresis, each band is cut out and electroeluted. A simple method to prepare PsaC is also presented.

Key Words: Photosystem (PS) I; preparation; reaction center (RC); subunits.

1. Introduction

Methods to prepare certain photosystem (PS)I reaction center subunit polypeptides are described. For PsaC, PsaD, PsaE, and PsaY, the filtrate resulting from the ultrafiltration in Chapter 6 is used for preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For PsaA and PsaB, Type II PSI particles, prepared as described in Chapter 2, are treated with SDS, then applied on preparative SDS-PAGE. After electrophoresis, each band is cut out and electroeluted.

2. Materials

1. Type II preparation described in Chapter 2.
2. The filtrate containing the small subunit described in Chapter 6.
3. Equipment for concentrating protein (*see* **Note 1**).
4. SDS-PAGE apparatus with a gradient gel maker (*see* **Note 2**).
5. An electroelution apparatus for acrylamide gel (*see* **Note 3**).
6. Elution buffer: 7.5 *M* urea, 0.1% SDS and 400 m*M* Tris-HCl, pH 8.8.

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7. Stacking gel: 4.87% acrylamide, 0.13% methylene-*bis*-acrylamide, 0.125 *M* Tris-HCl buffer, pH 6.8, 7.5 *M* urea and 0.1% SDS.
8. Separating gel: 10% acrylamide, 0.13% methylene-*bis*-acrylamide, 7.5 *M* urea, 0.1% SDS and 400 m*M* Tris-HCl, pH 8.8.

3. Methods

3.1. PsaC, PsaD, PsaE, and PsaY

The starting material is the first filtrate obtained in the ultrafiltration step described in Chapter 6.

3.1.1. Electrophoresis and KCl Staining

1. Preparative SDS-PAGE is performed as described in Chapter 2, except for the larger sized gel (*see* **Note 2**).
2. Prior to applying to the sample well, the filtrate must be concentrated as the amount applicable to one lane is limited to approx 10 µL (*see* **Note 3**). Use as many lanes as available.
3. After electrophoresis, the removed gel slab is immersed in 2 *M* KCl filled in a shallow dish.
4. After a few minutes, the gel turns translucent except for protein bands, which remain transparent. Cut out protein bands by using a disposable surgical knife.

3.1.2. Electroelution of the Bands

The gel pieces of the protein bands are placed in an electroeluter filled with the elution buffer. The elution is performed according to manufacturer's instruction.

3.2. PsaA and PsaB

1. The starting material is either Type II or Type III preparation.
2. SDS-PAGE is performed as described above except for the gel system. Use the specific stacking and separating gel solutions mentioned in material section, **Subheading 2., items 7** and **8**, and a prolonged running time.
3. Prior to injection, the filtrate must be concentrated.
4. It is important to continue electrophoresis after BPB front line passes through the end of the gel, and continue running for several hours (*see* **Note 4**).
5. After electrophoresis, the gel slab is KCl-stained, and the bands are cut out and electroeluted as stated above.
6. A typical separation pattern is shown in **Fig 1**.

3.2.1. Characterizations

These subunit proteins are tentatively identified from relative mobilities on SDS-PAGE. Positive identification requires either N-terminal sequencing or mass spectroscopy (*see* **Note 4**).

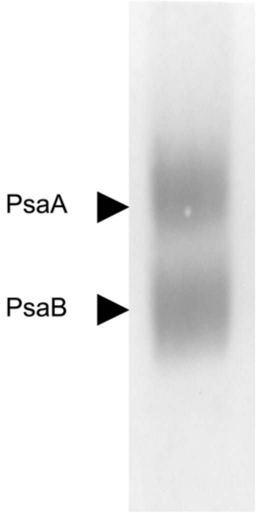

Fig. 1. A special SDS-PAGE of photosystem I particles to achieve PsaA/PsaB separation. Electrophoresis was performed as described in the text.

4. Notes

1. Numerous devices have been on the market. A Centricon (Millipore) is an example of such device.
2. For preparation, a large gel size (higher than 15-cm) is recommended. Although a thicker gel up to 2 mm can be used to get better yield, a thinner gel usually gives better resolution.
3. Numerous types of equipment have been on the market. A Centrilutor Micro-Electroeluter (Millipore), has been used in our laboratory for some years.
4. Some of the subunit proteins, such as PsaA, PsaB, and PsaL, cannot be sequenced as a result of amino-terminal blocking. Matrix-assisted laser desorption induced time-of-flight mass spectroscopy (MALDI-TOF-MS) can be applied to protease-treated samples.
5. A simplified yet effective preparation for PsaC: The filtrate is passed through a BioRad Econo-Pac CHT-II (1-mL type) that has been equilibrated with a urea medium (7.5 *M* urea, 10 m*M* Tris-HCl, pH 7.8, 0.1% Triton X-100 and 0.1% 2-mercaptoethanol) and washed once with 10 mL of the medium. Passed-through and washed-out fractions are combined and concentrated by ultrafiltration. A purified preparation of PsaC can be obtained this way ***(1)***.
6. For other subunit proteins, less purified PSI preparations, such as digitonin-particles ***(2)***, are needed. SDS-PAGE followed by electroelution described above may be utilized for this purpose.

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9

Purification and Crystallization of the Cytochrome b_6f Complex in Oxygenic Photosynthesis

Huamin Zhang and William A. Cramer

Summary

The cytochrome b_6f complex from the thermophilic cyanobacterium *Mastigocladus laminosus* and spinach chloroplasts has been purified as a dimeric species. It was found by electrospray ionization mass spectroscopy to contain eight and nine subunits, respectively, and dimeric masses of 217,070 and 286,454 Da. The subunits common to the complex from both sources are petA (cytochrome *f*), B (cytochrome b_6), C (Rieske iron-sulfur protein), D (subunit IV), and small 3.2–4.2 kDa polypeptides petG,L,M, and N. The ninth polypeptide, the 35 kDa petH polypeptide in the spinach complex, was identified as ferredoxin NADP reductase (FNR), which binds to the complex tightly at a stoichiometry of approx 0.9 (cyt f)$^{-1}$. The spinach complex contains diaphorase activity diagnostic of FNR, and is active in facilitating ferredoxin-dependent electron transfer from NADPH to the cytochrome b_6f complex. The purified cytochrome b_6f complex contains stoichiometrically bound chlorophyll *a* and β-carotene at a ratio of one per cytochrome *f*, and bound lipid, in which MGDG and PG are the most abundant species. The delipidated highly purified complexes are active immediately after preparation and for approx 1 wk if left on ice, transferring 300–350 electrons/cyt *f*/*s*. Both complexes are subject to proteolysis and associated loss of activity if left for extended periods (>1 wk) at room temperature. Addition of pure synthetic lipid to the delipidated *M. laminosus* complex (the "lipid augmentation" technique) allows rapid and ready formation of large (>0.2 mm) crystals suitable for x-ray diffraction analysis and structure determination, which diffract with good statistics to 3.0 Å.

Key Words: Cytochrome (cyt); dimethyl sulfoxide (DMSO); ethylenedinitrilo tetraacetic acid (EDTA); ferredoxin:NADP$^+$ oxidoreductase (FNR); monogalactosyl-diacylglycerol (MGDG); phosphatidylglycerol (PG); plastoquinone bound on electrochemically negative side of b_6f complex (Q_n); *n*-undecyl-β-D-maltoside (UDM).

1. Introduction

The hetero-oligomeric cytochrome b_6f complex (plastoquinol:plastocyanin/cytochrome c_6 oxidoreductase) functions in oxygenic photosynthesis to mediate electron transfer from photosystem (PS)II to PSI in the linear electron transfer

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pathway ***(1–3)***, or around PSI in a cyclic pathway ***(4)***, generating an electrochemical proton gradient across the membrane that is used for the synthesis of adenosine 5'-triphosphate. The b_6f complex is an integral membrane protein complex composed of eight or nine polypeptide subunits ***(4,5)***, and is an obligate dimer ***(6,7)***. The complex contains three traditional hemes per monomer (two linked to the cytochrome *b* polypeptide ***(8)***, and one covalently attached to cytochrome *f*), one novel covalently bound heme, "heme X" in cytochrome b_6 ***(8a)***, and two non-heme Fe in the prosthetic group of the Rieske iron-sulfur protein (ISP). The b_6f complex also mediates transmembrane activation of the stromal-side light-harvesting complex protein kinase ***(9)***.

The complex is phylogenetically analogous to the cytochrome bc_1 complex of mitochondria and photosynthetic bacteria, for which crystal structures have been obtained from bovine ***(10–13)***, avian ***(11)***, yeast ***(14,15)***, and bacterial ***(16)*** sources. In cyanobacteria, the b_6f complex is shared by both photosynthetic and respiratory electron transfer chains because of the absence of the cytochrome bc_1 complex ***(1)***. The 24-kDa cytochrome b_6 subunit, which has four transmembrane α-helices and contains two b-type hemes, together with the 17-kDa subunit IV, which has three transmembrane α-helices, are functionally and structurally analogous to the N-terminal and C-terminal helices of the cytochrome *b* in the bc_1 complex ***(8)***. The 19-kDa Rieske ISP, which has an N-terminal single transmembrane α-helix domain and a C-terminal 140 residue soluble extrinsic domain including a linker region connecting these two domains, is structurally and functionally related to the ISP subunit in the bc_1 complex ***(17, 18)***. The 31-kDa *c*-type cytochrome *f* subunit is functionally but not structurally related to cytochrome c_1 in the bc_1 complex ***(17,19)***. Among the *c*-type cytochromes, the β-strand secondary structure and distal heme ligation by the N-terminal α-amino terminus of cytochrome *f* are unique structural signatures that are conserved from the cyanobacteria ***(20)*** through the green algae ***(21,22)*** to plants ***(17,19)***.

It is of significant interest to compare the high resolution structure of the b_6f complex with that of bc_1. Among the questions that could be answered by the existence of a high-resolution structure for b_6f are:

1. Does the unique structure of the cytochrome *f* soluble domain imply a unique structure-function relation?
2. Does the insensitivity of the hinge region of the Rieske protein to mutational perturbation ***(23)*** imply a different set of conformations compared to that in the bc_1 complex ***(10–13)***?
3. What is the conformation of the quinone bound on electrochemically negative side of b_6f complex (Q_n) site that precludes the action of Q_n-type quinone analog inhibitors such as antimycin A that are active toward the bc_1 complex?

4. What is the function of the chlorophyll *a* ***(6,24,25)*** and β-carotene ***(26)*** that are unique to *b_6f* compared to *bc_1* complexes?

2. Materials

1. Sources of the complex: Spinach and the thermophilic cyanobacterium, *Mastigocladus laminosus*. Spinach was purchased from the market. Fresh leaves were washed and stored at 4°C. The thermophilic cyanobacterium *M. laminosus* was grown in 10 L carboys at 55°C to late logarithmic phase, and harvested, 4 carboys at a time, utilizing a Millipore Pellicon™ filtration system.
2. Cell breakage solution for *M. laminosus* cells: 25 m*M* Hepes/KOH, pH 7.5, 0.4 *M* sucrose, 10 m*M* $MgCl_2$, 10 m*M* $CaCl_2$.
3. Homogenizing solution for spinach leaves (TNS): 50 m*M* Tris-HCl, pH 7.5, 0.1 *M* NaCl, 0.2 *M* sucrose.
4. Propyl-agarose (Sigma).
5. Solid *n*-undecyl-β-D-maltoside. Stored at –20°C.
6. Solid *n*-octyl-β-D-glucoside (Anatrace). Stored at –20°C.
7. Buffer 1: 10 m*M* Tricine/NaOH, pH 8.0, 2 *M* NaBr, 0.3 *M* sucrose.
8. 20 m*M* Tricine/NaOH, pH 8.0.
9. TMKNE buffer: 30 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 5 m*M* KCl, 50 m*M* NaCl, 1m*M* EDTA.
10. Buffer 2: 50 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA, 0.05% UDM.
11. Buffer 3: 50 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA. 0.05% UDM, 20% DMSO.
12. Buffer 5: 30 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 0.05% UDM.
13. Buffer 7: 100 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$ and 30% PEG 400.
14. 8% (w/v) sucrose in TMKNE, 0.05% *n*-undecyl-β-D-maltoside.
15. 40% (w/v) sucrose in TMKNE, 0.05% *n*-undecyl-β-D-maltoside.
16. Sample buffer for SDS-PAGE: 50 m*M* Tris-HCl, pH 8.6, 8 *M* urea, 4% SDS, 10% glycerol, and 4% mercaptoethanol.

3. Methods

3.1. Thylakoid Membrane Preparation

3.1.1. Spinach

1. Homogenize one bushel of leaves in a Waring 4-L blender for 2 min in 1.5 liters TNS.
2. Filter the slurry through a wire sieve and then through four layers of cheesecloth.
3. Centrifuge the eluate at 13,000*g* in a Sorvall GSA rotor for15 min.
4. Suspend the sediment in 1.2 L of 0.15 *M* NaCl solution, homogenize, and sediment at 13,000*g* for 15 min.
5. The pellet was resuspended in 1.2 L of buffer 1.
6. The suspension was stirred on ice for 20 min, an equal volume of cold water added, and then centrifuged at 13,000 *g* for 15 min.

7. This step was repeated, and thylakoid membranes then washed with 1.2 L of 0.15 *M* NaCl.

3.1.2. M. Laminosus

1. Harvest 40 g (wet weight) *M. laminosus* cells from 40 L culture, broken by shearing twice in a French press at 20,000 psi in 200 mL of cell breakage solution.
2. Sediment broken cell fragments by centrifugation at 250,000*g* in a Ti70 (Beckman) rotor for 45 min.
3. Resuspend thylakoid membranes and centrifuge in the following solution: 200 mL of 20 m*M* Tricine/NaOH, pH 8.0; 100 mL of buffer 1.
4. Stir the suspension on ice for 20 min.
5. Add an equal volume of cold water and centrifuge again at 300,000*g* for 45 min.
6. Repeat this step once.
7. Resuspend the pellet in 200 mL TMKNE buffer, and centrifuge at 300,000*g* for 45 min.

3.2. *Purification of the Cytochrome* b_6f *Complex*

3.2.1. Selective Solubilization of the Complex

1. Resuspend thylakoid membranes in TMKNE buffer to obtain a chlorophyll concentration of 2 mg/mL.
2. Slowly add an equal volume of TMKNE containing 60 m*M* OG, 0.1% cholate to the stirred membrane suspension.
3. Stir the mixture at room temperature for 15 min and centrifuge at 300,000*g* for 45 min.
4. Collect the supernatant, add solid ammonium sulfate to 35% saturation, stir at room temperature for 15 min, and then centrifuge at 300,000*g* for 30 min.
5. Collect the supernatant for further purification.

3.2.2. Propyl-Agarose Chromatography

1. Pack the propyl-agarose resin (Sigma) into a 2 × 15 cm column, wash sequentially with distilled water, TMKNE buffer, and equilibrate with 10 column volumes of TMKNE containing 35% (w/v) saturated ammonium sulfate, and 0.05% UDM.
2. Load the sample on the column by gravity.
3. Wash the column thoroughly with equilibration buffer until the eluant is colorless.
4. Elute the cytochrome b_6f complex with TMKNE containing 20% (w/v) saturated ammonium sulfate, 0.05% UDM. The earlier fractions were greenish, and those eluting later greenish-blue.
5. The cytochrome *f* concentration was determined by the difference spectrum of ascorbate minus ferricyanide, using an extinction coefficient of 30 mM^{-1} cm^{-1} at 554 nm relative to a baseline drawn between the spectral troughs at 538 and 568 nm *(27)*.
6. Fractions with a cytochrome *f* concentration greater than 1 μ*M* were pooled, and concentrated with a Centriprep 10 (Millipore) (*see* **Note 4**).

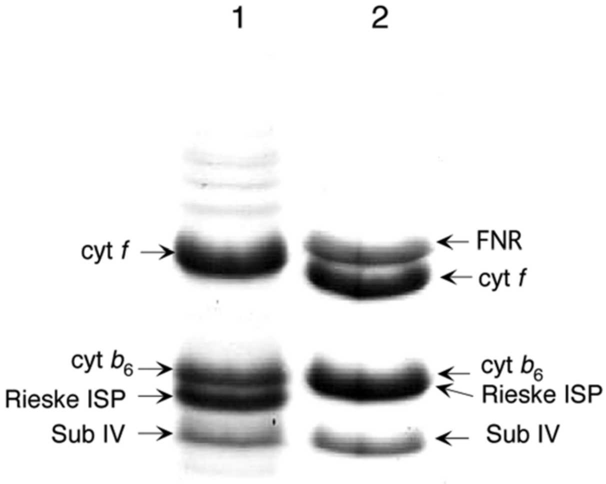

Fig. 1. SDS-PAGE of the cytochrome *b$_6$f* complexes from *M. laminosus* (lane 1) and spinach chloroplasts (lane 2). The four large (>17 kDa) subunits are labeled.

3.2.3. Sucrose Gradient Ultra-Centrifugation

1. Prepare a continuous sucrose gradient of 8–40% in TMKNE, 0.05% UDM.
2. Load the concentrated sample from the propyl-agarose column to the top of the gradient at a ratio of approx 1:10 (v/v).
3. Perform centrifugation at 160,000*g* in an SW-41 rotor for 16 h.
4. Collect the brown band in the middle of the gradient.
5. A second sucrose gradient is needed if the bands are not well separated in the first run.
6. After purification, store the cytochrome *b$_6$f* complex on ice in buffer 2.
7. Add synthetic dioleolyl-phosphatidylcholine (Avanti) to the complex at a final concentration of 0.1% (w/v) by diluting a 20% (w/v) stock solution stored in buffer 3.

3.3. SDS-PAGE

1. The sample was solubilized in the sample buffer, and then heated at 90°C for 2 min. The 15% SDS-gel was prepared according to the Laemmli system *(28)*.
2. The purified cytochrome complexes from spinach chloroplasts and *M. laminosus* show different patterns on SDS-PAGE. The four large subunits were well separated in the *M. laminosus complex* (**Fig. 1**, lane 1). However, in the spinach complex, *cyt b$_6$* and the Rieske ISP co-migrated into one thick band (**Fig. 1**, lane 2). An extra-high-molecular-weight band was detected in the spinach complex, which was characterized as a bound FNR *(4)*.

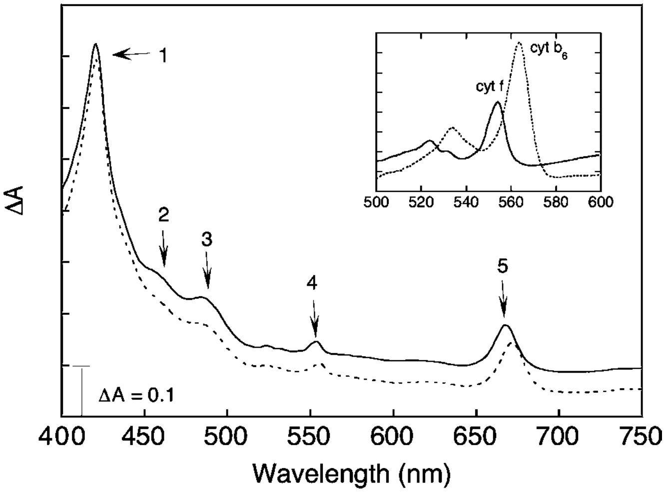

Fig. 2. Absolute visible absorbance spectra of the cytochrome b_6f complex from spinach chloroplasts (solid line) and *M. laminosus* (dashed line). The complexes (equivalent to 1 μ*M cyt f*) were reduced with Na-ascorbate. Peak 1, Soret bands; 2, FNR; 3, FNR and carotenoid; 4, cytochrome *f* α-band; 5, chlorophyll *a* Q_y band. Inset panel shows the spectra of *cyt f* and *cyt* b_6, respectively as ascorbate minus ferricyanide and dithionite minus ascorbate difference spectra.

3.4. Visible Difference Absorbance Spectra

1. The cytochrome content was determined from the difference spectra of the cytochrome complex.
2. The complex was diluted in 4 to 0.1 mg/mL buffer.
3. Visible spectra were measured on a Cary 3 UV-visible spectrophotometer with a measuring beam half-band width of 2.0 nm.
4. The absorbance spectrum of the complex was measured as a difference spectra (ascorbate minus buffer; **Fig. 2**, outset panel).
5. Cytochrome *f* and b_6 spectra were measured, respectively, as difference spectra, ascorbate minus ferricyanide and dithionite minus ascorbate (**Fig. 2**, inset panel).

3.5. Electron Transfer Activity

1. Electron transfer activity was measured according to the method of Black et al. *(29)*. The assay mixture contained 5 μ*M* plastocyanin, 5 n*M* cytochrome complex in buffer 4.
2. Reduction of plastocyanin was initiated by addition of 25 μ*M* decyl-plastoquinol, and monitored by the absorbance change at 600 nm relative to that at 500 nm.
3. The electron transfer activity of the isolated cytochrome complex from *M. laminosus* is typically 200–300 electrons/cyt f/s.

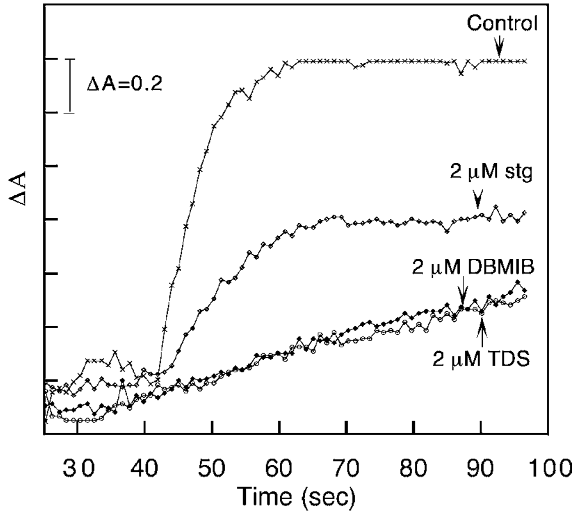

Fig. 3. Electron transfer activity and inhibitor effects. The electron transfer activity of the isolated cytochrome complex from *M. laminosus* is typically 200–300 electrons/cyt f/s. In the presence of 2 μ*M* stigmatellin, the activity was inhibited approx 60%. Whereas inhibition by 2 μ*M* tridecyl-stigmatellin or 2 μ*M* DBMIB is >90%.

4. In the presence of 2 μ*M* stigmatellin, the activity was inhibited approx 60%, whereas inhibition by 2 μ*M* tridecyl-stigmatellin or 2 μ*M* DBMIB is >90% (**Fig. 3**).

3.6. Mass Spectrometry

The subunit composition of the cytochrome complex was analyzed by mass spectrometry *(5)*. The active dimeric forms of the cytochrome complex from the cyanobacterium, *Mastigocladus laminosus*, and spinach chloroplasts contain eight and nine subunits, respectively, which are petA (cytochrome *f*), petB (cytochrome *b*), petC (Rieske ISP), petD (subunit IV), and the small 3.2–4.2 kDa polypeptides petG, L, M, and N, as listed in **Table 1**. The ninth subunit in the spinach complex was identified as ferredoxin:NADP+ reductase (FNR).

3.7. Lipid Analysis

Total lipids were extracted from the isolated complex according to the procedure described by Sato and Murata *(30)*.

1. Thin-layer chromatography was performed on a precoated 20 × 20 cm silica gel plate (Merck).
2. 50 μL of extracted lipid solution was applied to the plate.

Table 1
Masses and Subunits of the Cytochrome b_6f Complex

	Measured Mass (Da)	
Protein	*Spinacea oleracea*	*Mastigocladus laminosus*
FNR	35314	
PetA (*Cyt f*)	31935	32270
PetB (*Cyt* b_6)	24887	24710
PetC (Rieske iron-sulfur protein)	18936	19295
PetD (subunit IV)	17312	17528
PetG	4198	4057
PetM	3972	3841
PetL	3478	3530
PetN	3197	3304
Total Mass of Monomer	143227	108535

3. The plate was developed in $CHCl_3/CH_3OH/NH_4OH$ (28%) = 13/7/1.
4. The lipids were visualized by spraying 50% H_2SO_4 in methanol, followed by heating at 110°C until the spots are visible.
5. Standard lipids were run side by side with the sample.
6. The lipid content was determined by densitometric analysis of the scanned image using NIH Image 1.54.

The major bound lipids found in the less pure cytochrome complex are MGDG and PG, which are the most abundant lipids in cyanobacterial thylakoid membranes ***(30)***. However, in the extensively purified complex, there was less than one bound MGDG lipid per *cyt f.*

3.8. Pigment Analysis

The pigment content of the purified complex was determined as described by Zhang et al. ***(26)***. The isolated cytochrome complex contains stoichiometrically bound chlorophyll *a* and β-carotene at a ratio of approximately one per monomer.

3.9. Crystallization of the Cytochrome b$_6$f Complex

1. The cytochrome complex was concentrated with a Centricon 100 (Millipore) to 20 mg/mL.
2. Crystallization droplets were created by the hanging drop vapor diffusion technique at 18°C. 2 µL of concentrated protein (20 mg/mL) in buffer 4 was mixed with an equal volume of buffer 5.

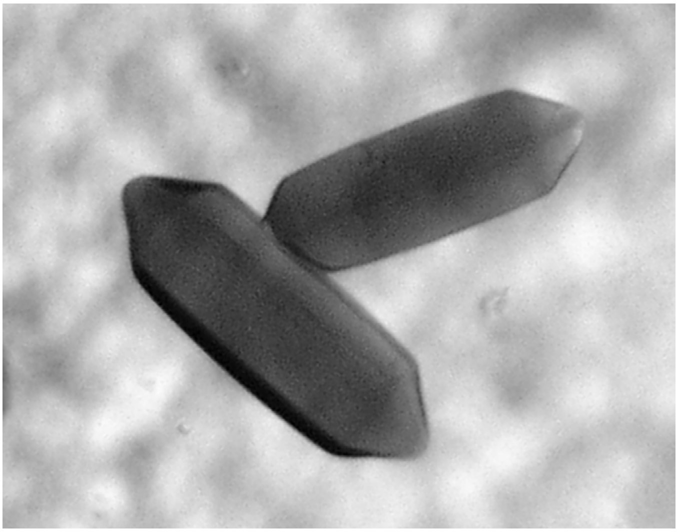

Fig. 4. Crystals of the cytochrome b_6f complex from *M. laminosus.* The reddish brown hexagonal crystals (0.5 × 0.25 × 0.2 mm) with space group $P6_1$ diffracted to 3.0 Å with good statistics ***(31)***.

3. Hexagonal crystals with space group $P6_1$ grew overnight to 0.3 × 0.15 × 0.1 mm, and reached a size of 0.5 × 0.25 × 0.2 mm after 3–4 d (**Fig. 4**).
4. For data collection, crystals were frozen in 28% PEG 400 with 5% 2, 3-butanediol.

4. Notes

1. All of the stock solutions should be stored at 4°C in the presence of protease inhibitors: 0.25 m*M* PMSF, 2 m*M* benzamidine, 2 m*M* α-amino-caproic acid.
2. In the present chapter we have described the methods for purification of the cytochrome b_6f complex from spinach chloroplasts and a thermophilic cyanobacterium. For purification of the complex from the green alga, *Chlamydomonas reinhardtii*, consult Pierre et al. *(7)*.
3. The purified cytochrome complex is subject to proteolysis. Even in the presence of protease inhibitors, the Rieske ISP, cytochrome b_6 and subunit IV could be partially proteolysed if left at room temperature for a week or so. After 2 wk at room temperature, these three subunits were completely cleaved (**Fig. 5**). The complex is stable on ice over several weeks, and it can be frozen in liquid nitrogen and stored at –80°C for a longer period of time without significant loss of activity.
4. After elution, the propyl-agarose column was still blue because of bound phycobilin proteins. The column can be regenerated by washing with one volume of distilled water, followed by seven sequential washes with one volume of 25, 50, and 95% ethanol, two volumes of *n*-butanol, and one volume of 95, 50, and 25% ethanol. The clean resin is washed and kept in distilled water.

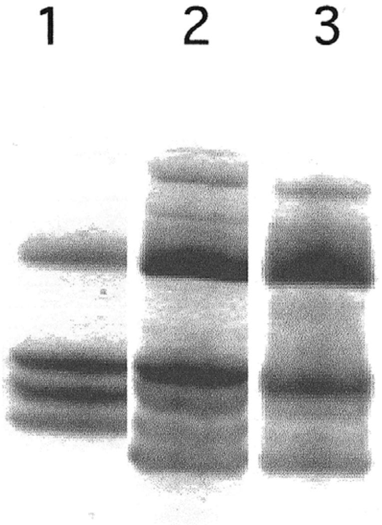

Fig. 5. Proteolysis of the cytochrome b_6f complex. Lane 1, fresh complex; lanes 2 and 3, incubated at room temperature for 7 and 14 d, respectively.

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10

Purification of Plastocyanin and Cytochrome c_6 From Plants, Green Algae, and Cyanobacteria

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Summary

Plastocyanin and cytochrome c_6 are widely distributed over the oxygen-evolving photosynthetic organisms. The two proteins are functionally equivalent, but strongly differ in their global electrostatic charge. In fact, they are acidic in eukaryotes, but either neutral or basic in cyanobacteria. The difference in their electrostatic features is a critical factor in designing the purification procedure, which must be modified and adapted accordingly. This chapter reports the methods for producing (including cell cultures), isolating and purifying plastocyanin and cytochrome c_6—which greatly differ in their isoelectric point—from a number of eukaryotic and prokaryotic organisms.

Key Words: Cyanobacteria; cytochrome c_6; *Escherichia coli*; green algae; metalloproteins; *Monoraphidium*; *Nostoc*; plastocyanin; protein purification; recombinant protein; spinach; *Synechocystis*.

1. Introduction

Plastocyanin and cytochrome c_6 are two soluble metalloproteins located inside the thylakoidal lumen. They have different tertiary structures—the former is a β-barrel protein with a copper atom, whereas the latter is an iron protein consisting of four α-helices and a heme group—but play the same physiological role: the transfer of electrons between the two membrane-embedded complexes cytochrome b_6f and photosystem I. Plastocyanin and cytochrome c_6 are alternatively synthesized in green algae and cyanobacteria in response to copper availability, although plastocyanin seems to be constitutively produced in plants. The molecular mass (approx 10 kDa) and redox potential (approx 350 mV, pH 7.0) of these two proteins are near each other. However, their global electrostatic

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charge varies from organism to organism but is the same within each organism. In fact, the two proteins are acidic in eukaryotes (plants and green algae) but neutral or highly basic in prokaryotes (cyanobacteria) ***(1)***. The purification procedure of any given protein (either plastocyanin or cytochrome c_6) must thus be modified depending on the organism but is the same for the two proteins isolated from the same source. The variable content in DNA, pigments, and other cellular components of cyanobacteria, green algae, and plants is a crucial point. In this chapter, we describe the methods for the production, isolation, and purification of plastocyanin and cytochrome c_6 from a number of organisms with different isoelectric points (pI): spinach and *Monoraphidium* (pI 3.5–4), Synechocystis (pI approx 6) and Nostoc (pI approx 9).

2. Materials

2.1. Organisms

Spinach (*Spinacea oleracea*) plants can be obtained from a local market. The use of freshly harvested plants improves neither the quality nor the yield of plastocyanin preparations.

The green alga *Monoraphidium braunii* can be obtained from the Culture Collection of Algae at the University of Göttingen (www.gwdg.de/~epsag), with the accession number 202-7d.

The cyanobacteria *Synechocystis sp* and *Nostoc sp*. (formerly *Anabaena sp.*) can be obtained either from the Pasteur Culture Collection (PCC, Institute Pasteur, 28 rue du Dr. Roux, 75724, Paris Cedex 15, France; www.pasteur.fr/recherche/banques/PCC), with the accession numbers 6803 and 7119, respectively, or from the American Type Culture Collection (ATCC, PO Box 1549, Manassas, VA 20108; www.atcc.org), with the accession numbers 27184 and 29151, respectively.

Escherichia coli (*E. coli*) transformable strains TG1 (phenotype: *sup*E *hsd*Δ5 thiΔ (*lac-pro*AB) F' [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*Z Δ*M15*]) ***(2)*** and RV308 (phenotype: *su*$^-$ Δ*lac*$_x$74 *gal*ISII::OP308 *strA*; ATCC 31608) are used for the expression of spinach plastocyanin. *E. coli* transformable strain MC1061 (phenotype: F$^-$ *ara*D139 Δ(*ara-leu*)7696 *galE15 galK16* Δ(*lac*)X74 *rpsL* (Str^r) *hsdR2* (r_K– m_K+) *mcrA mcrB1*) ***(3)*** are used for the expression of cyanobacterial proteins. Alternatively, other strains can be used ***(4)***.

2.2. Culture Media

Sterile culture media can be prepared in either glass Roux flasks, glass Erlenmeyer flasks, or glass cylindrical bottles (10 and 20 L). When bubbling is required, the gas flow is passed through commercial 0.2 μm filters of different sizes depending on culture volume. Foaming is avoided by adding one or two drops of a suitable antifoaming agent (e.g., silicone) to every liter of culture medium before sterilization. The specific compositions of the different culture media for green algae, cyanobacteria, and enterobacteria are described in the following subheadings.

1. *Monoraphidium*: the culture medium for *Monoraphidium braunii* cells: 14.5 m*M* NaH_2PO_4, 2.5 m*M* Na_2HPO_4, 1 m*M* $MgSO_4$, 10 m*M* KNO_3, 0.7 μM $ZnSO_4$, 2.5 μ*M* $MnCl_2$, 10 μ*M* Na_2MoO_4, 0.1 m*M* $CaCl_2$, 8 μ*M* H_3BO_3 and 8 m*M* NaCl ***(5)***. In order to avoid salts precipitation, the medium should be prepared from a sterilized and 100-fold concentrated solution containing all the components but the phosphates. After dilution of the stock solution, solid $NaH_2PO_4{\cdot}H_2O$ (2 g/L) and $Na_2HPO_4{\cdot}12H_2O$ (0.9 g/L) are added just before sterilization. Iron is added (0.5 mL to every liter of culture medium) from a complex Fe(III)-EDTA solution that is prepared as follows: 16 g of EDTA and 10.4 g of KOH are dissolved in 186 mL of distilled water, and 13.7 g of $FeSO_4{\cdot}7H_2O$ are dissolved in 364 mL of distilled water; the two solutions are mixed and vigorously bubbled overnight with air in the dark to fully oxidize iron atoms. To make the cells synthesize plastocyanin, 10 μ*M* $CuSO_4$ must be added to the culture medium.
2. *Synechocystis* and *Nostoc*: Cyanobacterial cells can grow in the BG11 medium (without copper), which contains 0.2 m*M* Na_2CO_3, 0.3 m*M* $MgSO_4$, 0.24 m*M* $CaCl_2$, 0.2 m*M* K_2HPO_4, 28.5 μ*M* citric acid, 6 mg/L of iron(III)-ammonium citrate (Fe 17%), 2.4 μ*M* Na_2-EDTA, 46 μM H_3BO_3, 9.1 μ*M* $MnCl_2$, 1.6 μ*M* Na_2MoO_4, 0.8 μ*M* $ZnSO_4$ and 0.2 μ*M* $CoCl_2$ ***(6)***. To avoid salt precipitation, the culture medium is prepared from a 100-fold concentrated stock solution containing all components but K_2HPO_4. After dilution of the stock solution, phosphate is added from a solution of 1*M* K_2HPO_4. Solid $NaNO_3$ (1.5 g/L) and $NaHCO_3$ (1.0 g/L) are added just before sterilization. To make the cells synthesize either cytochrome c_6 or plastocyanin, the content of iron(III)-ammonium citrate is doubled or 1 μ*M* $CuSO_4$ is added, respectively.
3. *Escherichia coli*: *E. coli* cells can grow in the Luria-Bertani (LB) standard medium, which contains 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter ***(7)***. To maintain and amplify the plasmids, ampicillin is added to the LB medium at 100 μg/mL final concentration from a 1000-fold concentrated stock solution sterilized by filtration through a 0.25 μm sterile filter. The filtered ampicillin solution can be stored at –20°C for several months. The medium is used for the expression of cytochrome c_6, but it must be supplemented with 200 μ*M* $CuSO_4$ in case of plastocyanin.

2.3. Purification of Proteins

2.3.1. Spinach Plastocyanin

1. Buffer A: 0.4 *M* sucrose and 15 m*M* NaCl in 20 m*M* Tricine-KOH, pH 8.0.
2. Buffer B: 5 m*M* Tricine-KOH, pH 8.0.

3. 1 *M* Tris-HCl buffer, pH 7.3.
4. 10 m*M* potassium phosphate buffer, pH 7.0.
5. 10 m*M* Tris-HCl buffer, pH 8.0.
6. 10 m*M* Tris-acetate buffer, pH 8.0 (*see* **Note 1**).
7. Acetone 80% (v:v) in distilled water.
8. Reagent grade pyridine.
9. Protamine sulfate: 20 mg/mL in 20 m*M* Tricine-KOH, pH 8.0; make fresh as required.
10. Solid $MgCl_2$.
11. Solid NaCl.
12. 0.1 *M* potassium ferricyanide in distilled water.
13. Washed DEAE-cellulose.
14. Polybuffer Exchanger 74.
15. Polybuffer 74 solution.
16. Amicon pressure filtration cells (250 and 10 mL) with YM-3 membranes.
17. Dialysis membranes (3.5 kDa cut-off, several diameters).
18. Three chromatography columns (6 × 12 cm, 2 × 15 cm and 1.5 × 20 cm).
19. Gradient glasses (three couples of 1, 0.4, and 0.125 L).
20. Waring blender (1 gallon) or similar apparatus.
21. Teflon-glass homogenizer (50 mL).
22. Branson 250 sonicator (or equivalent) with a standard tip.
23. Organdy or cheesecloth (a piece of 1 × 1 m).

2.3.2. Monoraphidium *Plastocyanin and Cytochrome* c$_6$

1. Buffer C: 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* benzamidine and 1 m*M* ε-aminocaproic acid in 20 m*M* phosphate, pH 7.0 (make fresh as required).
2. 10 m*M* Tris-HCl buffer, pH 8.0.
3. 10 m*M* Tris-acetate buffer, pH 8.0 (*see* **Note 1**).
4. Streptomycin sulfate: 0.1 *M* in water, pH 7.0; make fresh as required.
5. Solid ammonium sulfate.
6. Solid NaOH.
7. Solid NaCl.
8. 0.1 *M* potassium ferricyanide in distilled water.
9. Washed DEAE-cellulose.
10. Polybuffer Exchanger 74.
11. Polybuffer 74 solution.
12. Amicon pressure filtration cells (250 and 10 mL) with YM-3 membranes.
13. Dialysis membranes (3.5 kDa cut-off, several diameters).
14. Two chromatography columns (2 × 20 cm and 1.5 × 20 cm).
15. Gradient glasses (two couples of 0.4 and 0.125 L).
16. Manton-Gaulin disruptor (or similar apparatus).
17. Teflon-glass homogenizer (50 mL).
18. Branson 250 sonicator (or equivalent) with a standard tip.

2.3.3. Synechocystis Plastocyanin and Cytochrome c$_6$

1. Buffer C: 1 m*M* PMSF, 1 m*M* benzamidine and 1 m*M* ε-aminocaproic acid in 20 m*M* phosphate, pH 7.0 (make fresh as required).
2. 2 m*M* Tris-HCl buffer, pH 8.0.
3. 5 m*M* Tris-acetate buffer, pH 8.0 (*see* **Note 1**).
4. 20 m*M* Tricine-KOH buffer, pH 7.5.
5. Streptomycin sulfate (0.1 *M* in water, pH 7.0; make fresh as required).
6. Solid ammonium sulfate.
7. Solid NaOH.
8. Solid NaCl.
9. 0.1 *M* potassium ferricyanide in distilled water.
10. Washed DEAE-cellulose.
11. Polybuffer Exchanger 94.
12. Polybuffer 74 solution.
13. Amicon pressure filtration cells (250 and 10 mL) with YM-3 membranes.
14. Dialysis membranes (3.5 kDa cut-off, several diameters).
15. Two chromatography columns (2 × 30 cm and 1.5 × 20 cm).
16. A Superdex 75 (Amersham) FPLC column (16 × 60 cm) (or equivalent).
17. Gradient glasses (two couples of 0.4 and 0.125 L).
18. Manton-Gaulin disruptor (or some other similar apparatus).
19. Branson 250 sonicator (or some other equivalent) with a standard tip.

2.3.4. Nostoc *Plastocyanin and Cytochrome* c$_6$

1. Buffer C: 1 m*M* PMSF, 1 m*M* benzamidine and 1 m*M* ε-aminocaproic acid in 20 m*M* phosphate, pH 7.0 (make fresh as required).
2. 1 m*M* phosphate buffer, pH 7.0.
3. 20 m*M* Tricine-KOH buffer, pH 7.5.
4. Streptomycin sulfate: 0.1 *M* in water, pH 7.0; make fresh as required.
5. Solid ammonium sulfate.
6. Solid NaOH.
7. Solid NaCl.
8. 0.1 *M* potassium ferricyanide in distilled water.
9. Washed carboximethyl-cellulose.
10. Amicon pressure filtration cells (250 and 10 mL) with YM-3 membranes.
11. A chromatography column (2 × 30 cm).
12. A Superdex 75 (Amersham) FPLC column (16 × 60 cm) (or equivalent).
13. Gradient glasses (0.4 L).
14. Manton-Gaulin disruptor (or similar apparatus).
15. Branson 250 sonicator (or equivalent) with a standard tip.

3. Methods

The protocols have been modified from a series of previously reported methods for the isolation and purification of plastocyanin and/or cytochrome c_6 from

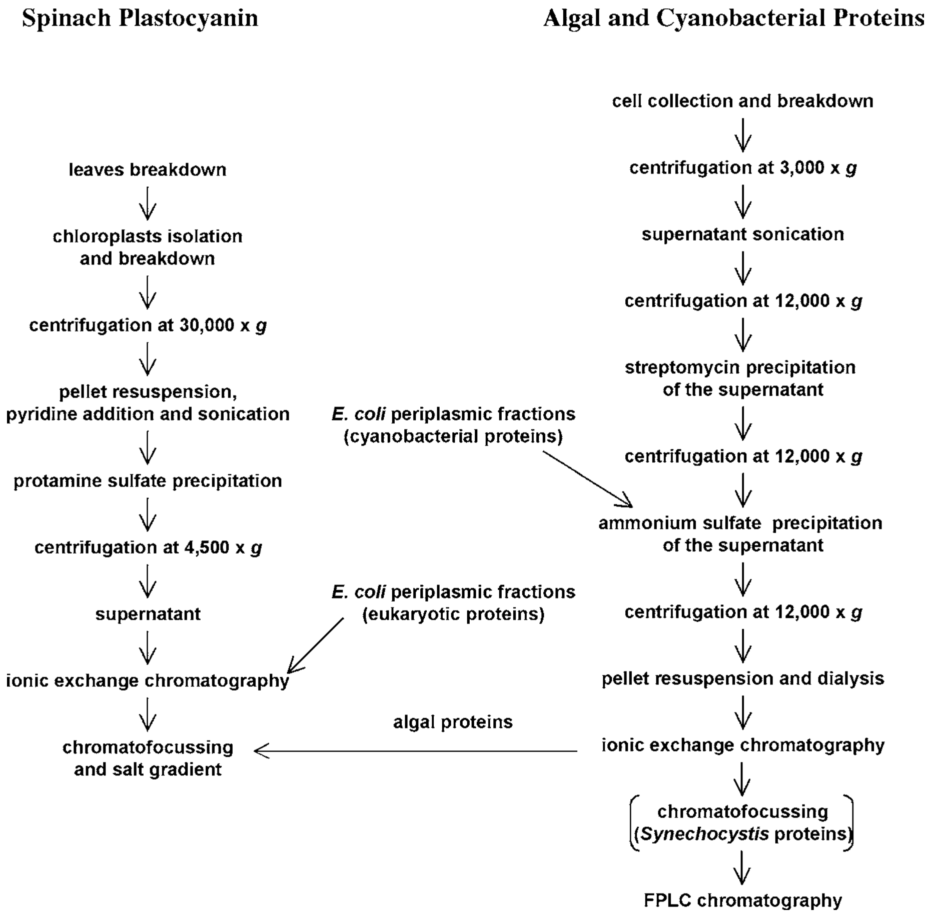

Fig. 1. Flow-chart summarizing the different steps of the procedures for purification of plastocyanin and/or cytochrome c_6 from plants, green algae, and cyanobacteria.

spinach *(8,9)*, *Monoraphidium* ***(10,11)***, *Synechocystis* ***(12,13)***, and *Nostoc* ***(14–15)***. **Figure 1** shows a flow-chart with the different procedures.

3.1. Cell Growth and Collection

3.1.1. Green Algae and Cyanobacteria

Grow green algal and cyanobacterial cells in culture rooms at 25 or 30°C, respectively. Routinely maintain cells by 1/10 dilution of stationary cultures (absorbance at 600 nm higher than 1.5) in sterile glass Roux flasks (culture volume, 750 mL) (*see* **Note 2**). Inoculate a 10 L sterile glass bottle, which in turn serves to inoculate four 20 L glass bottles. All the cultures are under continuous irradiation by fluorescent light tubes (50 µE m^{-2} s^{-1}) and gas bubbling (air:CO_2 mixture, 99/1 [v/v]). Collect cells from 80 L cultures at stationary phase (absorbance at 600 nm, 1.6–1.8) by centrifugation at 14,000*g* in either a continuous flow system or large volume rotor. Freeze centrifuge tubes with cell pellets overnight for efficient recovery of the pellets. Typical cell yields are around 1–2 g (wet weight)/L. The pellets can be kept at –20°C until use.

3.1.2. Escherichia Coli

For high yield protein purification, *E. coli* cells are transformed by following the standard molecular biology methods ***(9,13,15)***. Inoculate 100 µL of transformed cells in a 250 mL glass Erlenmeyer flask containing 100 mL of LB medium. After 12 h growing at 37°C under continuous stirring in an orbital shaker (200 rpm), the culture is used to inoculate three 5 L glass Erlenmeyer flasks containing 2 L of LB medium each. Leave cells to grow under similar temperature and shaking conditions to reach the stationary phase (approx 18 h; absorbance at 600 nm higher than 1.2), and collect upon centrifugation at 14,000*g* for 5 min. For systems under control of the T7 promoter, isopropyl-β-D-thio-galactopyranoside (IPTG) at a final concentration of 0.5–1 m*M* is added to the culture 8 h before cell collection (absorbance at 600, approx 1.0). Freshly collected cells are immediately used for protein purification. All cultures are made under sterile conditions.

3.2. Cell Breaking Procedures

3.2.1. Spinach Leaves

The starting material consists of 3 kg of depeciolated and denerved spinach leaves that must be washed with cold distilled water and dried in a cold chamber. This is a time-consuming procedure, thus material should be prepared the day before and kept in a cold room in the dark.

1. 500 g batches of spinach leaves are mixed with 700 mL of buffer A and broken in the Waring blender (30 s of blending at 70% power).
2. The resulting suspension is filtered through four organdy or cheesecloth layers in a funnel to eliminate unbroken material.
3. To recover as much crude extract as possible, the cell suspension has to be squeezed by hand.
4. The filtered suspension is centrifuged at 300*g* for 1 min to remove whole cells, and the supernatant is again centrifuged at 4000*g* for 10 min to precipitate chloroplasts.
5. The resulting pellet is resuspended with a glass bar in 400 mL of buffer B, and gently homogenized in a teflon-glass homogenizer.
6. The suspension is left to stand for 10 min under gentle stirring in darkness and then centrifuged at 30,000*g* for 20 min.

7. The final pellet is resuspended in 200 mL of distilled cold water and homogenized again.
8. Chlorophyll concentration is determined as described by Arnon ***(16)***: 0.1 mL of suspension is dissolved in 20 mL of 80% acetone in water (v/v).
9. After vigorous mixing, unsoluble material is removed upon centrifugation at 13,000*g* for 5 min at room temperature.
10. The absorbance at 652 nm of the supernatant is measured, and the chlorophyll content is determined by using an extinction coefficient of 34.5 mg chlorophyll mL^{-1} cm^{-1}.

3.2.2. Green Algal and Cyanobacterial Cells

1. The initial steps are identical for *Monoraphidium, Synechocystis, and Nostoc* cells. Mix 250 g of frozen cell paste with 1 L of cold buffer C (freshly prepared, owing to the unstability of the protease inhibitors) and keep under vigorous stirring at 4°C for at least 1 h in the dark.
2. Cell disruption is carried out on pressure/depressure cycles by passing the cell suspension three times through a Manton-Gaulin high pressure continuous system at 7000 psi (*see* **Note 3**).
3. The resulting suspension is centrifuged at 3000*g* for 2 min.
4. The supernatant is sonicated in 150 mL aliquots in an ice bath for 1 min with a Branson apparatus (50% power) after adding solid NaCl at 50 m*M* final concentration.
5. The suspension is centrifuged at 12,000*g* for 20 min, and the pellet is discarded.

3.2.3. Escherichia Coli *Cells*

E. coli transformed cells are collected upon centrifugation of 6 L cultures, and the pellets (*see* **Subheading 3.1.2.**) are resuspended in 20 mL of distilled water. Transformed cells are designed to send the overexpressed proteins (either plastocyanin or cytochrome c_6) to the periplasmic space, therefore the cell suspension has to undergo two cycles of freezing in liquid nitrogen and thawing at 40°C in a thermostatted bath. Upon centrifugation at 12,000*g* for 5 min, the periplasmic fraction is collected from the supernatant.

3.3. Purification of Plastocyanin and Cytochrome c_6

The purification procedures must be carried out in a cold chamber (4°C) or, alternatively, the cell and protein samples are kept in ice containers. To better follow the proteins throughout purification, cytochrome c_6 is maintained in its reduced native redox state so that it can be easily recognized by its typical pink-orange color, whereas a few microliters of a 0.1 *M* potassium ferricyanide stock solution are added to plastocyanin to get the characteristic blue color of its oxidited state (*see* **Note 4**). From here thereafter, the same protocol is used for purification of plastocyanin and cytochrome c_6 when they are both isolated from the same organism. Purity of cytochrome c_6 fractions is determined from the A_{553}/A_{275} ratio of its reduced state (*see* **Note 5**), whereas purity of plastocyanin samples is determined from the A_{278}/A_{597} ratio of its oxidized state. Plastocyanin is in part autoreduced throughout purification, therefore its redox state should be checked at every step by measuring the absorbance change at 597 nm in the absence of ferricyanide and after addition of a little amount of oxidant.

3.3.1. Spinach Plastocyanin

3.3.1.1. DIFFERENTIAL PRECIPITATION

1. Chlorophyll and buffer concentration of the suspension obtained in **Subheading 3.2.1.** is first fitted to 1.5 mg (chlorophyll)/mL and 10 m*M* Tris-HCl, pH 8.0, respectively, by adding distilled water and 1 *M* Tris-HCl, pH 8.0.
2. Add pyridine (0.7 mL to every 100 mL of solution) and gently stir the mixture for 30 min.
3. Sonicate the resulting solution (150 mL aliquots each time) in a Branson apparatus (100% power) for 150 s in a salt-ice bath to avoid sample heating.
4. Add a protamine sulfate solution (20 mg/mL in 20 m*M* Tricine-KOH, pH 8.0) under vigorous stirring to reach the proportion of 1 mL of protamine solution to every 5 mL of sonicated solution.
5. Stir the resulting preparation for 5 min and allow to rest for 30 min.
6. Add solid $MgCl_2$ under continuous stirring up to reach a final concentration of 5 m*M*.
7. After 10 min with no stirring, the preparation is centrifuged at 14,000*g* for 15 min and the pellet is discarded.

3.3.1.2. ANIONIC EXCHANGE CHROMATOGRAPHY

1. Load the supernatant resulting from the previous step onto a DEAE-cellulose column (6 × 12 cm; 300 mL of gel volume; elution flow ca. 40 mL/h) that must be previously equilibrated with 10 m*M* potassium phosphate, pH 7.0.
2. After sample loading, wash the column with at least 600 mL of the phosphate buffer to remove unbound material.
3. Elute the plastocyanin by applying a 0.01–0.4 *M* NaCl gradient in phosphate buffer (total volume, 2 L).
4. Pool plastocyanin fractions with an A_{278}/A_{597} ratio lower than 10, fully oxidize with potassium ferricyanide and dialyze for 24 h against 10 m*M* Tris-HCl, pH 8.0, and supplement with 10 μ*M* ferricyanide, with at least one dialysis buffer replacement.
5. Load the resulting dialyzed preparation—or, alternatively, the supernatant obtained from *E. coli* transformed cells in **Subheading 3.2.3.** onto a second DEAE-cellulose column (2 × 15 cm; 40 mL of gel volume; elution flow ca. 20 mL/h), which must be previously equilibrated with 10 m*M* Tris-HCl, pH 8.0.
6. After sample loading, wash the column with at least 80 mL of the same Tris-HCl buffer supplemented with 60 m*M* NaCl to remove weakly bound material.

7. Plastocyanin is eluted by applying a 0.06–0.3 *M* NaCl gradient in Tris-HCl buffer (total volume, 0.4 L).
8. Plastocyanin fractions with an A_{278}/A_{597} ratio lower than 5 are pooled, dialyzed against 10 m*M* Tris-acetate, pH 8.0, and fully oxidized with ferricyanide.

3.3.1.3. CHROMATOFOCUSING

1. The solution resulting from the previous step is loaded onto a chromatofocusing (Polybuffer Exchanger 94) column (1.5 × 20 cm, 30 mL of gel volume, elution flow ca. 10 mL/h) that must be previously equilibrated with 10 m*M* Tris-acetate, pH 8.0.
2. Wash the column with 150 mL of a 10-times diluted polybuffer 74 solution, pH 4.0, and elute plastocyanin by applying a 0.01–0.45 *M* NaCl gradient in 25 m*M* Tris-acetate, pH 5.0 (total volume, 0.2 L).
3. Fractions with an A_{278}/A_{597} ratio close to 1 are pooled, suspended in an adequate buffer upon several cycles of concentration/dilution in an Amicon pressure filtration cell (YM-3 membrane), and finally frozen until use.

3.3.2. Monoraphidium *Plastocyanin and Cytochrome* c6

3.3.2.1. DIFFERENTIAL PRECIPITATION

1. Add streptomycin sulfate (0.1 *M* in water, pH 7.0) to the supernatant obtained in **Subheading 3.2.2.** at the final ratio of 1/10 (v/v) under vigorous stirring.
2. Gently stir the solution for 1 h and allow to rest for 2 h.
3. Discard the precipitated material upon centrifugation at 12,000*g* for 20 min.
4. Slowly add solid ammonium sulfate to the supernatant, to reach a saturation level of 60% (36.1 g added to every 100 mL), under vigorous stirring and continuous pH testing to keep the pH value close to 7.0 by adding small amounts of a concentrated NaOH solution.
5. Gently stir the resulting preparation for 1 h and then clear upon centrifugation at 12,000*g* for 20 min.
6. Add ammonium sulfate to the supernatant as before to reach a saturation level of 98% (27.5 g added to every 100 mL).
7. Upon centrifugation at 12,000*g* for 20 min, resuspend the pellet by means of a glass bar in 200 mL of 10 m*M* Tris-HCl, pH 8.0, and dialyze against the same Tris-HCl buffer for 48 h with at least two dialysis buffer replacements.

3.3.2.2. ANIONIC EXCHANGE CHROMATOGRAPHY

1. The dialyzed solution is loaded onto a DEAE-cellulose column (2 × 20 cm; 60 mL of gel volume; elution flow ca. 20 mL/h) that must be previously equilibrated with 10 m*M* Tris-HCl, pH 8.0.
2. After column washing with at least 120 mL of Tris-HCl buffer, elute plastocyanin or cytochrome c_6 by applying a 0.01–0.4 *M* NaCl gradient in Tris-HCl buffer (total volume, 0.6 L).

3. Pool and dialyze plastocyanin or cytochrome c_6 containing fractions.

3.3.2.3. CHROMATOFOCUSING

1. The solution resulting from the previous step is further purified by chromatofocusing as described under **Subheading 3.3.1.**
2. The pure protein fractions with either an A_{278}/A_{597} ratio of approx 1.5 for plastocyanin or an A_{553}/A_{275} ratio of 1.15 in case of cytochrome c_6 are pooled, suspended in an adequate buffer upon several cycles of concentration/dilution in an Amicon pressure filtration cell (YM-3 membrane), and finally frozen until use.

3.3.3. Synechocystis *Plastocyanin and Cytochrome* c_6

3.3.3.1. DIFFERENTIAL PRECIPITATION

1. Streptomycin and two-sequential ammonium sulfate precipitations are as described under **Subheading 3.3.2.**, with the two following exceptions:
   a. In case of proteins isolated from *Synechocystis*, the first ammonium sulfate precipitation is performed at a saturation level of 50% rather than 60%, and
   b. In case of recombinant proteins expressed in and isolated from *E. coli*, the streptomycin precipitation step is omitted and the supernatants obtained at **Subheading 3.2.3.** are directly subjected to sequential ammonium sulfate precipitation.

3.3.3.2. ANIONIC EXCHANGE CHROMATOGRAPHY

1. After 98% ammonium sulfate precipitation, resuspend the centrifugation pellets with a glass bar in 200 mL of 2 m*M* Tris-HCl, pH 8.0, desalt along several cycles of concentration/dilution with the same buffer in an Amicon pressure filtration cell, and finally dilute with buffer up to 400 mL final volume.
2. Load the resulting solution onto a DEAE-cellulose column (2 × 30 cm, 80 mL of gel volume; elution flow ca. 20 mL/h) that must be previously equilibrated with 2 m*M* Tris-HCl, pH 8.0.
3. Wash the column with at least 80 mL of Tris-HCl buffer.
4. Plastocyanin or cytochrome c_6 are eluted from the column by applying a 0-0.2 *M* NaCl gradient in Tris-HCl buffer (total volume, 0.6 L), and the fractions with an A_{275}/A_{597} (plastocyanin) or A_{274}/A_{552} (cytochrome c_6) ratio lower than 10 are pooled and dialyzed against 5 m*M* Tris-acetate, pH 8.0.

3.3.3.3. CHROMATOFOCUSING

The solution resulting from the previous step is further purified by chromatofocusing as described in **Subheading 3.3.1.**, except that plastocyanin or cytochrome c_6 are eluted with 10-times diluted polybuffer 74 solution, pH 4.0. The fractions with an A_{275}/A_{597} lower than 5 (plastocyanin) or an A_{274}/A_{552} ratio lower than 3 (cytochrome c_6) are pooled and concentrated by pressure filtration in an Amicon cell.

3.3.3.4. FPLC CHROMATOGRAPHY

1. The protein solution resulting from the previous step is subjected to FPLC chromatography on a Superdex 75 column (1.6 × 60 cm; elution flow 0.5 mL/min) previously equilibrated with 20 m*M* Tricine-KOH, pH 7.5.
2. Plastocyanin or cytochrome c_6 are eluted using the same Tricine-KOH buffer.
3. Pure protein fractions with an A_{275}/A_{597} ratio of approx 2.2 (oxidized plastocyanin) or an A_{552}/A_{274} ratio of 1.14 (reduced cytochrome c_6) are pooled, concentrated and frozen until use.

3.3.4. Nostoc *Plastocyanin and Cytochrome* c_6

3.3.4.1. DIFFERENTIAL PRECIPITATION

1. Streptomycin and two-sequential ammonium sulfate precipitations are as described under **Subheading 3.3.2.**
2. In case of recombinant proteins expressed in and isolated from *E. coli*, the streptomycin precipitation step is omitted and so the supernatants obtained at **Subheading 3.2.3.** are directly subjected to sequential ammonium sulfate precipitation.

3.3.4.2. CATIONIC EXCHANGE CHROMATOGRAPHY

1. After 98% ammonium sulfate precipitation, the centrifugation pellets are resuspended with a glass bar in 200 mL of 1 m*M* sodium phosphate, pH 7.0, and dialyzed against the same buffer for 48 h, with at least two dialysis buffer replacements.
2. The dialyzed solution is loaded onto a carboximethyl-cellulose column (2 × 30 cm; 80 mL of gel volume; elution flow ca. 20 mL/h) previously equilibrated with 1 m*M* sodium phosphate, pH 7.0.
3. Wash the column with at least 160 mL of phosphate buffer supplemented with 5 µ*M* ferricyanide.
4. Plastocyanin or cytochrome c_6 are eluted from the column by means of a 1–35 m*M* phosphate gradient, pH 7.0 (total volume, 0.6 L).
5. The fractions with an A_{275}/A_{597} (plastocyanin) or A_{274}/A_{552} (cytochrome c_6) ratio lower than 3 are pooled and concentrated.

3.3.4.3. FPLC CHROMATOGRAPHY

1. The protein solution resulting from the previous step is subjected to FPLC chromatography as described under **Subheading 3.3.3.**
2. Pure protein fractions with an A_{275}/A_{597} ratio of approx 1.2 (oxidized plastocyanin) or an A_{552}/A_{274} ratio of approx 1.0 (reduced cytochrome c_6) are pooled, concentrated, and frozen until use.

4. Notes

1. 10 m*M* Tris-acetate buffer, pH 8.0, must be prepared by adding small aliquots of concentrated acetic acid to a solution of 10 m*M* Tris to reach a pH value of 8.0.

2. For long-term maintenance, the organisms can be kept in either Petri dishes with solid media (1.5% [p/v] agar concentration) or 100 mL glass Erlenmeyer flasks with liquid medium and no bubbling. In the first stages of diluted cultures in glass Roux flasks, light intensity should be dimmed to avoid photobleaching by placing a piece of paper between flasks and fluorescent tubes. Every 80 L culture usually takes four days to reach the saturation level; after the second day, additional side panels of fluorescent lamps can be placed to enhance cell growing. Cell collection from each 80 L culture takes 4–5 h by continuous flow centrifugation.
3. The cells can alternatively be disrupted in a French press after three cycles at 20,000 psi, but there is a limit in the capacity of the cylinder (approx 40 mL). Algal (*Monoraphidium*) and cyanobacterial (*Synechocystis and Nostoc*) cells can also be broken in a Sorvall Omnimixer (or similar apparatus), the wet cell paste is carefully mixed with the same volume of 0.2 mm 0 glass beads; after 10 cycles of 30 s shaking with 2 min cooling intervals, the glass beads are separated upon decantation and the resulting suspension is centrifuged to remove unbroken cells. In case of *Synechocystis*, however, the cell wall has to be previously degraded by adding penicillin G (100 mg L^{-1}) to the cultures 24 h before collection.
4. Cyanobacterial plastocyanin cannot be followed throughout the first steps of purification because of optical interference of phycobiliproteins, and it is not until the ammonium sulfate fractionation that the copper protein can be detected.
5. Depending on the organism from which cytochrome c_6 is isolated, a difference of 1–2 nm in the maximum value of the ultraviolet (274–275 nm) and alpha (552–553 nm) bands can be observed in the absorption spectrum of the reduced form.

Acknowledgments

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11

Preparation of Native and Recombinant Light-Harvesting Chlorophyll-*a/b* Complex

Wolfgang Rühle and Harald Paulsen

Summary

Procedures to isolate native light-harvesting chlorophyll-*a/b* complex (LHCIIb) and to reconstitute recombinant LHCIIb are described. Separation of trimeric from monomeric forms and free pigment by sucrose density-gradient ultracentrifugation can be applied to both native and reconstituted complexes. The preparations are characterized by their pigment composition, protein pattern and spectral properties.

Key Words: *a/b*-Binding protein; antenna protein; LHCII; light-harvesting complex; vitro reconstitution.

1. Introduction

The apoprotein of the light-harvesting chlorophyll-*a/b* complex (LHCIIb) is the most abundant membrane protein in plants and one of the best-studied components of the photosynthetic apparatus. The latter is partially owing to the fact that LHCIIb is more stable and easily isolated than other pigment–protein complexes of the thylakoid membrane. The isolation procedure of Krupa et al. ***(1)*** described here is easy and rapid. Although the resulting LHCIIb preparation has a low content of violaxanthin, a weakly bound carotenoid easily lost from native LHCIIb ***(2)***, the complexes are well suited for various spectroscopic studies ***(3,4)*** and presumably also for crystallization **(5)**.

Studies of structure–function relationships in LHCIIb are facilitated by the fact that the protein can easily be refolded in vitro. This allows mutation or altered pigment composition in recombinant versions of the complex. A technique to reconstitute LHCIIb from its apoprotein and pigments in vitro has been devised by Plumley and Schmidt ***(6)*** and extended to bacterially expressed apoprotein ***(7)***. Subsequently, several slightly different procedures have been

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developed ***(8,9)***. In this chapter we present a broadly applicable procedure leading to preparative amounts of trimeric recombinant LHCIIb.

2. Materials

2.1. Native LHCIIb

1. *Pisum sativum* (*see* **Note 1**).
2. Homogenization buffer: 50 m*M* Tricine-NaOH (pH 7.8), 0.4 *M* sorbitol.
3. Washing buffer: 5 m*M* EDTA-NaOH (pH 7.8), 50 m*M* sorbitol.
4. Triton X-100, 5% (w/v) stock solution (especially purified for membrane research, Roche, Mannheim/Germany).
5. KCl, 1 *M*.
6. $MgCl_2$, 1 *M*.
7. Sucrose, 0.5 *M*.
8. Resuspension buffer: 50 m*M* Tricine-NaOH (pH 7.8), 100 m*M* sorbitol.

2.2. Recombinant LHCIIb

1. Bacterial strain expressing LHCIIb apoprotein Lhcb1*2 from pea ***(10,13)***.
2. Solubilization buffer: 200 m*M* Tris-HCl (pH 9.0), 4% (w/v) Li-dodecylsulfate (LDS), 10 m*M* ε-aminocapronic acid, 3 m*M* benzamidine, 25% (w/v) sucrose.
3. β-Mercaptoethanol, 1 *M*.
4. Octyl-β-D-glucopyranoside (OG) (Bachem, Heidelberg/Germany), 10% (w/v) stock solution.
5. OG-buffer: 1% (w/v) OG, 100 m*M* Tris-HCl (pH 9.0), 12.5% (w/v) sucrose.
6. TX-buffer: 0.05% (w/v) Triton X-100 0.01% (w/v) L-phosphatidyl-D. L-glycerol dipalmitoyl (PG) (Avanti Polar Lipids, Alabaster/AL), 100 m*M* Tris-HCl (pH 7.5).
7. Elution buffer: 0.05% (w/v) Triton X-100, 0.01% (w/v) PG, 10 m*M* Tris-HCl (pH 7.5), 300 m*M* imidazol.
8. Chelating Sepharose Fast Flow (Amersham Biosciences, Uppsala/Sweden).

2.3. Density Gradient

1. Gradient solution: 0.4 *M* sucrose, 0.06% β-D-dodecylmaltoside (Biomol, Hamburg/Germany), 10 m*M* Tris-HCl (pH 7.0).

3. Methods

3.1. Preparation of Native LHCIIb

Low-salt thylakoids are used as starting material for this procedure. Intrinsic proteins are solubilized by the non-ionic detergent Triton X-100, the LHCIIb is precipitated by K^+ and Mg^{2+} salts, and then separated from other proteins by sedimentation through a sucrose layer. The resulting pellet consists mainly of trimeric LHCIIb and can further be separated from contaminating monomeric LHCIIb and minor antenna complexes by density-gradient ultracentrifugation (*see* **Subheading 3.3.**).

3.1.1. Preparation of Low-Salt Thylakoids

All steps of thylakoid preparation are carried out at 4°C.

1. Homogenize approx 300 g of young pea shoots (*see* **Note 1**) in a Waring blender with one liter of ice-cold homogenization buffer.
2. Filter through four layers of cheesecloth and centrifuge for 5 min at 7000*g*.
3. Wash the pellet once with 200 mL cold washing buffer and centrifuge for 10 min at 10,000*g*.
4. Suspend thylakoids in cold distilled water to a chlorophyll *a+b* concentration of 0.8 mg/mL ***(11)*** (*see* **Note 2** for chlorophyll determination).

3.1.2. Solubilization and Precipitation of LHCIIb

1. Add Triton X-100 from a 5% (w/v) stock solution to the low-salt thylakoids to give a final detergent concentration of 0.67% (w/v) (140 µL 5% Triton per mL suspension; molar ratio detergent/chlorophyll = 12/1).
2. Incubate the suspension at room temperature in the dark for 30 min with continuous stirring.
3. Centrifuge for 40 min at 30,000*g* at 4°C.
4. Discard the pellet and add KCl as well as $MgCl_2$ from 1 *M* stock solutions in several portions to the supernatant to give final concentrations of 100 m*M* KCl and 20 m*M* $MgCl_2$ (4 µL KCl and 23 µL $MgCl_2$ per mL supernatant).
5. Gently stir the solution at room temperature for at least 10 min in the dark to precipitate the LHCIIb.
6. Prepare a cushion of 0.5 *M* sucrose in centrifuge tubes on which the suspension is carefully layered. The volume of the sucrose should be at least threefold the volume of the LHCIIb suspension.
7. Centrifuge at 10,000*g* for 10 min at 4°C (fixed-angle rotor sufficient).
8. Resuspend the pellet in resuspension buffer and bring the solution to a chlorophyll concentration of 0.8 mg/mL.
9. Add Triton X-100 to give a molar detergent/chlorophyll ratio of 10/1 (116 µL 5% Triton per mL suspension). Stir for 10 min at room temperature.
10. Repeat precipitation with KCl and $MgCl_2$ as above and centrifuge through a sucrose layer at 30,000*g* for 10 min. The chlorophyll *a/b* ratio should be below 1.4 after this step. Otherwise steps 8–10 should be repeated with centrifugation at 10,000*g* for 10 min.
11. Resuspend the pellet in resuspension buffer, add KCl and $MgCl_2$ as above but omit Triton.
12. Centrifuge through a sucrose layer for 10 min at 10000g.
13. Resuspend the pellet in a small volume of distilled water, spin down for 3 min at 5000g.
14. Repeat **step 13** to remove any soluble detergent and rests of salt.
15. Adjust the suspension to a chlorophyll *a+b* concentration of 0.8 mg/mL and store aliquots at –20°C or

16. Solubilize the native protein preparation for sucrose density-gradient ultracentrifugation of step 3.3 with 1% dodecylmaltoside at a chlorophyll concentration of 1 mg/mL for 5 min on ice. Remove nonsolubilized material by centrifugation at 15,000*g* for 2 min, and carefully layer the supernatant onto the gradient.

3.2. Preparation of Recombinant LHCIIb

The procedure starts out from recombinant Lhcb1*2 protein carrying a C-terminal hexahistidyl (His) tag ***(10,13)***. This protein is accumulated in the overexpressing bacteria as insoluble inclusion bodies. The pigments are isolated by acetone extraction of pea or spinach leaves. The procedure can be scaled up or down as needed. Recombinant LHCIIb, primarily formed as monomeric complexes, spontaneously oligmomerizes into trimers when detergents are removed in the presence of phosphatidyl glycerol ***(12)***. This is convenient when the His-tagged complexes are immobilized on Ni^{2+}-chelating affinity material ***(13)***.

3.2.1. Reconstitution of Recombinant Protein With Pigments

1. Dry an acetonic pigment extract (*see* **Note 3**) of pea or spinach leaves equivalent to 0.5 mg chlorophyll in a nitrogen stream and store aliquots for future use at –20°C until use.
2. Solubilize 250 µg recombinant Lhcb1*2 apoprotein (inclusion bodies) in 300 µL solubilization buffer, 210 µL water and 30 µL 1*M* β-mercaptoethanol.
3. Incubate for 1 min in a boiling water bath.
4. Dissolve a pigment extract corresponding to 0.5 mg chlorophyll in 60 µL ethanol.
5. Vortex the protein solution while adding the pigment solution and for at least another 30 s (*see* **Note 4**). Results are best when both protein and pigment solutions are at room temperature.
6. Add 75 µL octylglycoside from a 10% (w/v) stock solution to each vial, to form mixed micelles of dodecylsulfate and octylglycoside during a 10-min incubation at room temperature.
7. Add 75 µL of 2 *M* KCl and put solution on ice for 10 min to precipitate potassium dodecylsulfate.
8. Centrifuge at 4°C and 8000*g* for 5 min and incubate on ice for another 15 min.
9. Centrifuge at 4°C and 15,000*g* for 5 min. Aggregated pigments and precipitated *K*-dodecylsulfate are removed by this step from the supernatant containing octylglycoside micelles with reconstituted complexes and free pigment.

3.2.2. Trimerization of Reconstituted LHCIIb on a Ni^{2+}-Chelating Column

1. Fill a 2-mL polypropylene chromatography column with Chelating Sepharose to obtain a sepharose bed of about 0.3–0.5 mL.
2. Add 0.5 mL 0.1 *M* $NiCl_2$ solution (the sepharose turns to a light green color).
3. Add 1 mL 50 m*M* Tris-NaOH (pH 7.5) (the sepharose color changes to a light blue).
4. Equilibrate the column with 0.5 mL OG-buffer and transfer to 4°C.

5. Close the outlet of the column and mix the supernatant from the reconstitution experiment with the sepharose bed. Resuspend every 10 min for a total of 30 min to achieve complete binding of the protein to the Ni-surface of the sepharose (*see* **Note 5**).
6. Wash the column with 3 × 0.5 mL OG-buffer. The brownish color of the eluate does not originate from pheophytin but from the reduction of some of the Ni^{2+} ions by mercaptoethanol. This does not interfere with the preparation.
7. Add 2 × 0.5 mL TX-buffer to change the detergent and provide the PG necessary for trimerization.
8. Finally release the LHCIIb from the nickel-sepharose by imidazol in 0.5 mL elution-buffer and collect all droplets with dark green color. The eluate contains a mixture of monomeric and trimeric LHCIIb, which can be separated by sucrose density-gradient ultracentrifugation.

3.3. Separation of Monomeric and Trimeric LHCIIb

This procedure is suitable for both native and recombinant LHCIIb.

1. Prepare a sucrose density gradient by freezing the gradient solution in 12–14 mL ultracentrifuge tubes (e.g., for rotor SW40, Beckman, Coulter) at –20°C and thawing it slowly at 4°C.
2. Remove the upper 500 µL of the gradient to provide a sharp step between the gradient and the sample.
3. Carefully load the gradient with solubilized native or reconstituted recombinant LHCIIb.
4. Centrifuge at 200,000*g* for 16 h in a swing-out rotor (e.g., SW40, Beckman, Coulter).

Figure 1 shows an example for the separation of monomeric from trimeric LHCIIb and free pigment in a preparation of native LHCIIb by sucrose density-gradient ultracentrifugation.

3.4. Characterization of the Isolated LHCIIb

3.4.1. Pigment Content

Table 1 is an example of yields and chlorophyll *a/b* ratios as a rough measure of purity after three precipitation steps. Chlorophyll concentrations are calculated from the absorbance of acetonic extracts ***(11)***.

A more detailed analysis of the pigment composition is performed by extraction of the gradient band by 2-butanol and separation in a RP-18 HPLC column (Chromolith SpeedROD. Merck, Darmstadt/Germany) ***(7)***. **Table 2** shows the pigment composition of the trimeric LHCIIb band. Lutein is used as a reference since 2 luteins are attached to a LHCIIb-protein. Biochemical analyses of native LHCIIb reveals 14–15 chlorophylls whereas only 12 chlorophyll molecules are resolved in the crystal-data ***(5)***. Unfortunately, violaxanthin is almost completely lost in this preparation (*see* **Note 6**).

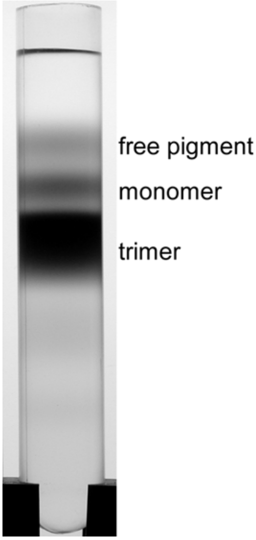

Fig. 1. Sucrose-density gradient separation pattern of a native LHCIIb preparation.

Table 1
Example of Yield and and Chlorophyll *a/b* Ratios
As a Rough Measure of LHCIIb Purity During Successive Precipitations

	Low salt thylakoids	P1= first precipitate	P2 = second precipitate	P3 = third precipitate
Total Chlorophyll [mg]	40.0	21.5	7.6	0.9
Yield [%]	100.0	53.7	19.0	2.1
Chlorophyll *a/b*	2.85	2.33	1.33	1.28

3.4.2. Protein Pattern

The purification is demonstrated in **Fig. 2** by a Coomassie-stained denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis. Lane **Thyl** results from thylakoids and lane **P1** from the first precipitation. After the second precipitation (**P2**) almost all other proteins except the apoproteins of LHCIIb and an unpigmented 65kD component are removed. However, silver-stained gels show that there are still traces of proteins other than LHCIIb apoproteins, which shows the limitation of the procedure. Salt precipitation is suitable for preparing large amounts of LHCIIb free of other pigment binding proteins within a short time but for further demands in purity other principles of separation like isoelectric focusing *(3)* are required.

Table 2
Pigment Stoichiometry of Native Trimeric LHCIIB.
Data are in mol/2 mol Lutein and Represent Means (± SD) of Nine Preparations

Nx	Vx	Lu	Chl*b*	Chl*a*	β-Car
0.8 ± 0.1	0.1 ± 0.1	2.0	6.4 ± 0.4	7.9 ± 0.7	—

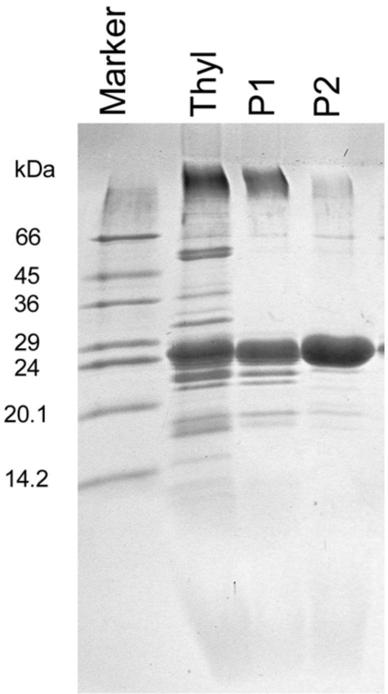

Fig. 2. Protein pattern at different steps of purification as revealed by a fully denaturing SDS-PAGE. **Thy**, thylakoids; **P1**, first precipitation; **P2**, second precipitation. A Laemmli gel ***(16)*** (15% polyacrylamide) was stained with Coomassie.

3.4.3. Spectroscopy

3.4.3.1. Absorption Spectrum

The room temperature absorption spectrum of LHCIIb has chlorophyll *a* maxima at 435 nm and 675 nm and chlorophyll *b* maxima at 471 nm and 650 nm as well as some shoulders in the blue region of the spectrum resulting from xantophylls (**Fig. 3**).

For low temperature absorption spectroscopy, the sucrose-gradient bands were mixed with an 80% glycerol solution buffered by 50 m*M* Hepes (pH 7.5)

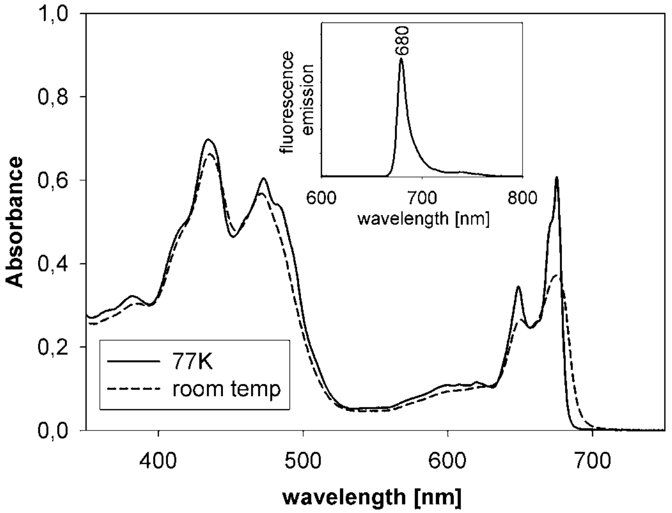

Fig. 3. Absorbance spectra of native trimeric LHCIIb at room temperature (dashed) and 77 K (solid). Insert: fluorescence emission spectrum of native trimeric LHCIIb at 77 K with 470 nm excitation.

to give a final glycerol content of 60% and subsequently measured in liquid nitrogen. Qy absorption forms of chlorophyll are clearly resolved at 650 nm, 660 nm, 670 nm, and 676 nm. The derivative spectra (not shown) reveal additional bands at 639 nm, 644 nm, and 656 nm. Further absorption bands in native and reconstituted LHCIIb have been reported to appear at 4 K *(4)*. The low temperature absorption spectrum of the reconstituted protein complex depends partially on the chlorophyll *a/b* ratio in the pigment extract used for the reconstitution. At a 3/1 ratio in the pigment extract the ratio of bound chlorophylls is about 1.0 instead of approx 1.2 in the native complex and the two long-wavelength absorbing forms at 670 nm and 676 nm are weaker than in the native complex *(9)*.

3.4.3.2. FLUORESCENCE EMISSION

Low temperature fluorescence spectroscopy was performed under the same conditions as absorption spectroscopy (insert in **Fig. 3**). Excitation at both 470 nm (Chl *b*) and 430 or 410 nm (Chl *a*) lead to an emission band at 680 nm (Chl *a*) indicating complete energy transfer from Chl *b* to Chl *a*. Significant formation of LHCIIb aggregates would be indicated by a fluorescence emission band at around 700 nm *(14)*.

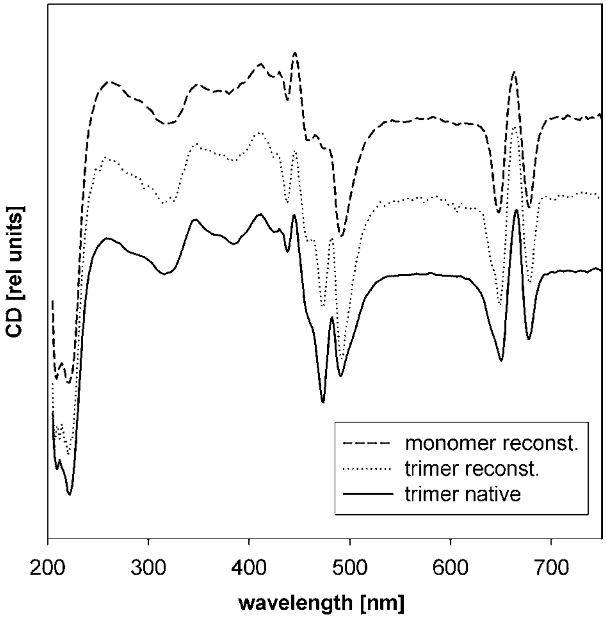

Fig. 4. CD spectra of monomeric reconstituted (dashed), trimeric reconstituted (dotted), and trimeric native LHCIIb (solid). All spectra are divided by their absorbance at 675 nm for normalization and displaced by 40 mdeg to separate them.

3.4.3.3. CD Spectrum

The UV region of CD spectra can provide interesting information on the secondary protein structure. Therefore it is necessary to remove the sucrose and Tris-HCl from the gradient-bands. This can be done either by repeated dilutions with a 10 m*M* phosphate buffer (pH 7.5), and subsequent ultrafiltration steps by 15 min centrifugations at 5000*g* with a 30 kDa size-exclusion membrane, or by buffer exchange on a G25 Sephadex gel filtration column. The CD spectrum (**Fig. 4**) of trimeric LHCIIb differs from the monomeric form by a significant shoulder at 642 nm ***(15)*** and a prominent minimum at 474 nm ***(12)*** the origin of which is still unclear. The bands between 200 nm and 240 nm present structural information on the amount of α helical secondary protein structure ***(9)***. There is almost no difference between the native and reconstituted LHCIIb.

4. Notes

1. The pea plants should be 10 to 14 d old. Keep them in darkness for 12 h before starting the preparations to prevent starch accumulation (avoid stems when harvesting).

2. Prepare an 80% acetonic extract of the thylakoids or protein complexes. Measure absorbance at 663.6 nm, 646.6 nm, and 750 nm. Subtract any absorbance at 750 nm from the 663.6 nm and 646.6 nm values. Calculate chlorophyll concentrations from Porra's ***(11)*** equations considering dilution factors:

$$\text{chlorophyll } a\ [\mu g/mL] = 12.25 \times A_{663.6} - 2.55 \times A_{646.6}$$
$$\text{chlorophyll } b\ [\mu g/mL] = 20.31 \times A_{646.6} - 4.91 \times A_{663.6}$$

3. The preparation of a total-pigment extract ***(7)*** is described briefly: extract a pellet of thylakoids (as prepared in **Subheading 3.1.1.**) with acetone and centrifuge at 5000*g* for 10 min. Partition the pigments into diethyl ether (0.25 volumes) by adding an equivalent volume of 1.3 *M* NaCl. Repeat ether extractions, combining the ether fractions, as long as the lower acetone/NaCl phase remains colored. Remove water from the ether fractions by freezing at –20°C and subsequent filtration. Evaporate the ether to dryness *in vacuo*. Solubilize the pigments in acetone, determine chlorophyll content ***(11)***, dry aliquots of 0.5 mg chlorophyll *a*+*b* in a nitrogen stream, and store at –20°C.
4. It is crucial to avoid local supersaturation of the pigments to bring them into the LDS micelles. Use vials large enough for vigorous vortexing without needing to close the lid. If 1.5 ml Eppendorf vials are used the 600 µL sample should be divided into three vials and combined after mixing or after the centrifugation **step 9** in **Subheading 3.2.1.**
5. If the column is equipped with a flow control, the sample can also slowly be loaded to the packed column within 30 min. The following washing and elution steps are as described. This will increase the binding capacity of the column and can lead to a higher LHCIIb concentration in the eluate.
6. Ruban et al. ***(2)*** published a procedure that yielded LHCIIb still containing substantial amounts of violaxanthin. They solubilize BBY particles or thylakoids with dodecylmaltoside and separate an "antenna band = A-band" by sucrose-gradient centrifugation. This A-band is then solubilized with low concentrations of dodecylmaltoside and separated with a second sucrose-gradient centrifugation. However this procedure is probably only suitable for analytical purposes because it has a very low yield.

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12

Isolation and Characterization of Lamellar Aggregates of LHCII and LHCII-Lipid Macro-Assemblies With Light-Inducible Structural Transitions

Ilian Simidjiev, Zsuzsanna Várkonyi, and Győző Garab

Summary

In this chapter we describe isolation of loosely stacked lamellar aggregates of LHCII that are capable of undergoing light-induced reversible structural changes, similar to those in granal thylakoid membranes (LHCII, the main chlorophyll *a/b* light-harvesting antenna complex of photosystem II). This unexpected structural flexibility of the antenna complexes depends largely on the lipid content retained during the isolation. The essence of the procedure is to adjust—for the plant material used—the proper conditions of detergent solubilization and purification mild enough for the associated lipids but also provide sufficient purity. Microcrystals and most other LHCII preparations, which are more delipidated, are not capable of similar changes. The light-induced structural reorganizations can be enhanced by the addition of different thylakoid lipids, which depending on the lipid species, also lead to the transformation of the lamellar structure. The preparation of different LHCII-lipid macro-assemblies is also described. In structurally flexible LHCII preparations and in thylakoids, the changes originate from a thermo-optic effect: fast local thermal transients, T-jumps, resulting from dissipation of the (excess) excitation energy that lead to elementary structural transitions in the vicinity of the dissipating centers. This can occur because thylakoids and structurally flexible LHCII assemblies but, for example, not the microcrystals, exhibit a thermal instability below the denaturation temperature, and thus (local) heating lead to reorganizations without the loss of the molecular architecture of the constituents. We also list the main biochemical and biophysical techniques that can be used for testing the structural flexibility of LHCII, and discuss the potential physiological significance of the structural changes in light adaptation and photoprotection of plants.

Key Words: Chloroplasts; dissipation; excess excitation energy; grana; LHCII; light adaptation; light-harvesting antenna; lipid–protein interactions; photoprotection of plants; structural flexibility; thermo-optic effect; thylakoid lipids.

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1. Introduction

1.1. Basic Observations

LHCII is the main, chlorophyll *a/b* light-harvesting antenna complex of photosystem (PS) II. It is the most abundant pigment-protein complex in the biosphere. Its structure, derived from electron crystallography of microcrystals, is known at near atomic resolution ***(1)***. The primary function of this complex is to absorb light and transfer the excitation energy toward the reaction centers. By mediating the stacking of granal thylakoid membranes ***(2)***, LHCII is also known to stabilize the granal ultrastructure; its high self-aggregation capability explains the lateral segregation (sorting) of the two photosystems between the granum and stroma membranes ***(3)***. LHCII also participates in multilevel regulatory processes, thermal, and light adaptation of plants ***(4,5)***.

LHCII, when isolated with mild detergent treatments ***(6,7)***, readily assembles in loosely stacked lamellar aggregates, which although less marked than in microcrystals, exhibit a significant degree of long range chiral order of their chromophores ***(8)*** and form-ordered two-dimensional (2D) arrays ***(9)***. These lipid-enriched preparations, in contrast to more delipidated samples, such as microcrystals, are capable of undergoing light-induced reversible structural reorganizations ***(10,11)*** that closely resemble the structural changes in the native thylakoid membranes ***(12)*** (*see* **Table 1**). In LHCII, the rate of these changes is linearly proportional to the light intensity. In thylakoids, the changes also exhibit a similar, approximately linear light-intensity dependence above the saturation of photosynthesis. Hence, this reversible reaction "measures" the light intensity meaning that the antenna system can react proportionally and reversibly to excess excitation i.e., above the saturation of photosynthesis.

1.2. Nature and Mechanism of the Structural Changes

As concerns from nature of the structural changes, a substantial degree of monomerization of LHCII has been shown to be involved both in vivo and in vitro. As for the physical mechanism, these reactions have been accounted for by a novel biological thermo-optic effect ***(13,14)***. As indicated by chlorophyll fluorescence kinetic analyses, the structural changes also bring about alterations in the photophysical pathways, most notably in the regulated quenching of the singlet excited state of chlorophyll *a* molecules in the light harvesting antenna ***(15)***. It seems likely that similar changes are responsible for the light-regulation of LHCII phosphorylation at the substrate level ***(16)***. Monomerization may also be important in the accessibility of LHCII to proteases ***(17)***. In general, local structural rearrangements in the antenna may provide the system with a structural flexibility without perturbing the structural stability of the membranes. The exact sequence of molecular events and the physiological significance of these changes remain to be elucidated in future studies.

Table 1
Comparison of Microcrystalline (Type IV) and Delipidated Disordered (Type III) LHCII Aggregates With Loosely Stacked Lamellar Aggregates (Type II) of LHCII and LHCII-Containing Thylakoid Membranes

Feature	LHCII microcrystals, delipidated aggregates	LHCII lamellar aggregates	Thylakoids	References
Long-range order of chromophores (psi type CD)	Very strong in microcrystals; none in delipidated aggregates	Strong (weaker than in microcrystals)	Strong	***7,8***
Light-induced reversible ΔCD, or other structural changes	None	Yes, fully reversible up to 500 Wm^{-2}	Yes, fully reversible up to 500 Wm^{-2}	***7,10,16, 19,20***
Lipid content (relative units)	60	330	500	***11***
Lipid enhancement of ΔCD	Not possible	Yes	No data	***11***
ΔF (reversible fluorescence quenching)	Weak	Strong	Non-photochemical quenching	***5,15,19–21***
Light-induced reversible Mg^{2+}-release	Not detectable	2.5 per trimer	Yes, non-saturable up to 500 Wm^{-2}	***14***
Thermal instability	High stability below 65°C	Breakdown of macrodomains: 40–50°C	Breakdown of macrodomains: 45–55°C	***13,14,20***
Light instability (>1000 $\mu E\ m^{-2}\ s^{-1}$, 15 min)	Very resistant at all temperatures up to 60°C	High instability (above 20–30°C)	High instability (above 25–30°C)	***13,14***

500 Wm^{-2} ~= 2700 $\mu E\ m^{-2}\ s^{-1}$ (of red photons).

1.3. LHCII-Lipid Macro-Assemblies

The light-induced structural changes depend largely on the presence of lipids *(7)*, and the addition of isolated thylakoid lipids substantially enhance the ΔCD *(11)*. Freshly isolated LHCII (Type II), prepared with the procedure described here, assembles in lamellar aggregates of loosely stacked sheets. This structure is retained after the addition of phosphatidylglycerol (PG) or sulfoquinovosyldiacylglycerol (SQDG). Upon the addition of digalactosyldiacylglycerol (DGDG), closed vesicles are formed, which stack to each other; upon the (gradual) addition of monogalactosyldiacylglycerol (MGDG), the loosely stacked lamellae are (gradually) transformed into onion-type structures or into stacked bundles of membranes *(11)*. When disordered large aggregates (that contain less lipids than lamellar aggregates but more than microcrystals) are to be restored by lipids, usually flat multilamellar arrays are obtained with all lipids, including MGDG *(9)*; these, however, are not capable of undergoing light-induced reversible rearrangements (as measured by ΔCD of their so-called ψ-type (psi, polymerization, and salt-induced) bands that originate from the long-range chiral order of the chromophores). (Trimer-monomer transition *[14]* may still be possible, but for that we have no systematic study yet.)

2. Materials

All buffers should be kept in refrigerator (4°C) and used within 1 mo.

2.1. Buffers

1. Buffer A: 50 m*M* Tricine, pH 7.8 (with NaOH), 0.4 *M* sorbitol.
2. Buffer B: 20 m*M* Tricine, 5 m*M* EDTA, pH 7.8 (with NaOH), 50 m*M* sorbitol.
3. Buffer C: 20 m*M* Tricine, pH 7.8 (with NaOH).
4. Buffer D: 0.5 *M* sucrose cushion equilibrated with 20 m*M* Tricine, pH 7.8 (with NaOH).
5. Buffer E: 50 m*M* Tricine, pH 7.8 (with NaOH) and 100 m*M* sorbitol.

2.2. Stock Solutions

1. 20% Triton-X 100 (Fluka) buffered with 20 m*M* Tricine, pH 7.8 (with NaOH). Store it at 4°C for up to 2 mo in dark brown bottle.
2. 2 *M* KCl.
3. 1 *M* $MgCl_2$.

3. Methods

3.1. Isolation of LHCII

We use pea or spinach leaves with good turgor and dark green color, but other plants might be equally good. For instance, Krupa et al. *(6)* used rye; our method is based on their procedure, as described previously *(7)*.

1. Take 50–60 g of fresh pea leaves (or approx 400 g of spinach), wash it with cold water, and then in chilled distilled water, put in dark at 4°C for at least 2 h. Homogenize with a mixer in 250 mL of buffer A (*see* **Note 1**).
2. Filter the homogenate through four layers of unused cheesecloth. Do not wash the cloth because detergent may influence the structure of the isolated LHCII.
3. Centrifuge at 5000*g* for 5 min.
4. Discard the supernatant and suspend the pellet in buffer B. Use medium size brush for crude homogenization of the pellet, and then use Potter homogenizer. Be sure that all the material is finely homogenized, without visible big particles (*see* **Note 2**).
5. Centrifuge at 10,000*g* for 10 min. Discard the supernatant (*see* **Note 2**).
6. Resuspend the pellet in cold buffer C to obtain an absorbance of about 1 in 0.2 mm cuvette at 680 nm, therefore, one can quickly adjust the chlorophyll content (*see* **Note 3**).
7. Add Triton-X 100 (Fluka), from the solution, to obtain final concentration of 0.7–0.9%. This is required for lamellar aggregates (Type II). To get LHCII microcrystals (Type IV—delipideted form), the final Triton concentration has to be 1.1–1.3% (*see* **Note 4**).
8. Stir on ice continuously for at least 45 min, until the visible clouds disappear. Continue stirring up to 60 min if clouds are present (*see* **Note 5**).
9. Centrifuge at 30,000*g* for 40 min. The insoluble material will be sedimented, and the supernatant will predominantly contain LHCII (*see* **Note 6**).
10. Discard the pellet and add to the supernatant 20–25 m*M* $MgCl_2$ and 100–150 m*M* KCl, from the stock solutions. Stir gently to precipitate the crude LHCII. It takes about 5 min to see clouds appearing in the suspension. Then, continue to stir for another 10–15 min (*see* **Notes 7** and **8**).
11. Layer the suspension on buffer D. In order to wash out the detergent; the volume of buffer has to exceed 3 times of the volume of the LHCII suspension. Centrifuge at 10,000*g* for 15 min.
12. Resuspend the pellet in buffer E to obtain an absorbance of 0.8–1.0 in 0.2 mm cuvette.
13. Add Triton-X 100 up to 0.2% final concentration, from 20% stock solution. Put the suspension in screw-cap tube, shake it; clouds will appear (best seen in reflected light). Shake until the clouds partially disappear (*see* **Note 9**).
14. Precipitate as in **step 10**. Stir suspension gently for approx 15 min.
15. Lay the suspension on sucrose cushion (buffer D) and centrifuge at 30,000*g* for 40 min.
16. For final purification suspend the pellet in buffer E and add 20 m*M* $MgCl_2$ and 100 m*M* KCl, from stock to reach these final concentrations. Triton is omitted in this step. Lay the suspension on 0.5 *M* Tricine-buffered sucrose cushion (buffer D) and centrifuge at 10,000*g* for 10 min. The final purification could be omitted if the chlorophyll *a/b* ratio is between 1 and 1.3.

    Then, transfer the solution to Eppendorf tube and centrifuged at 3000*g* for 5 min in Eppendorf centrifuge and resuspended in buffer C. This should be repeated three times. Finally the LHCII is homogenized again in buffer E and the chlorophyll concentration and chlorophyll *a/b* are measured according to Arnon *(18)*.

3.2. Preparation of LHCII-Lipid Macro-Assemblies

To obtain different thylakoid lipids, for LHCII incorporation, total lipid extraction from thylakoid membranes was performed according to Bligh and Dyer *(22)* following by separation of different lipid classes on thin-layer chromatography (TLC) plates according to the procedure of Vigh et al. *(23)*. The lipids were stored in hexane containing 0.05% bromhydroxytoluene (BHT).

The preparation of LHCII-lipid complexes was as follows:

1. Transfer the required lipid concentration to a sharp-conical glass tube and evaporate the hexane under argon or nitrogen stream.
2. Add buffer C to the dry lipid and fill the tube with nitrogen or argon, then close it with cap or Parafilm "M."
3. Vortex vigorously until the lipids are completely dissolved. Usually SQDG, PG, and DGDG are dissolved upon 4–5 min vortexing. During that time place the tube on ice several times for approx 30 s each time. Change vortexing and ice during the whole procedure of dissolving. Store the dissolved lipid mixture on ice.
4. To dissolve MGDG, add 0.05–0.08% Triton X-100 from the stock solution to buffer C. Then vortex vigorously for about 30 s, transfer the tube to ice for 30 s and sonicate the suspension in a water bath sonicator for another 30 s. Repeat these steps until all lipids are dissolved, usually four to five times.
5. Add the desired amount of LHCII preparation to the lipid mixture, fill the tube with nitrogen or argon, close it, and vortex mildly for about 20 s. Store the mixture on ice, and use it within 4 h.

3.3. Circular Dichroism (CD) Measurements

CD can be measured in a Jobin-Yvon CD6 dichrograph (**Fig. 1**). It is perhaps the easiest and quickest test for the quality of the preparation *(7)*.

The samples are usually placed at a distance of 5 cm from the photomultiplier. This can vary, but keeping the same geometry is important because of the involvement of differential scattering. Samples of 20 μg/mL chlorophyll content can be measured in a glass cuvette with 1 cm optical pathlength.

The presence of light-induced CD changes, ΔCD, is a clear indication of Type II LHCII. This can be measured with a side illumination attachment. In our measurements the samples were illuminated at 90° with respect to the measuring beam (**Fig. 2**). The actinic light from a 600 W tungsten halogen lamp passed through a heat filter (10 cm water) and 620 nm cutoff Corning 2-64 red filter and was directed to the sample with a light guide. Stray light from the actinic beam was blocked with a broadband Corning 4-96 blue filter.

Before CD measurements, LHCII samples were kept in the dark, at room temperature for a few minutes. CD was continuously monitored at 495 nm.

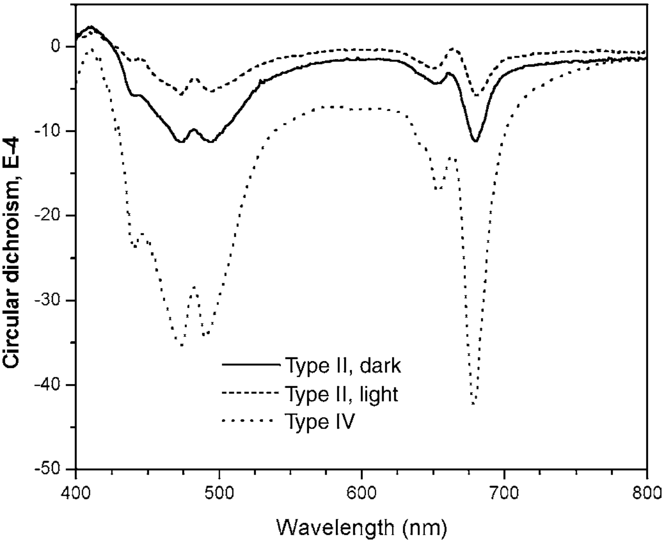

Fig. 1. Circular dichroism spectrum of loosely stacked lamellar aggregates of LHCII (Type II) and microcrystalline LHCII aggregates (Type IV).

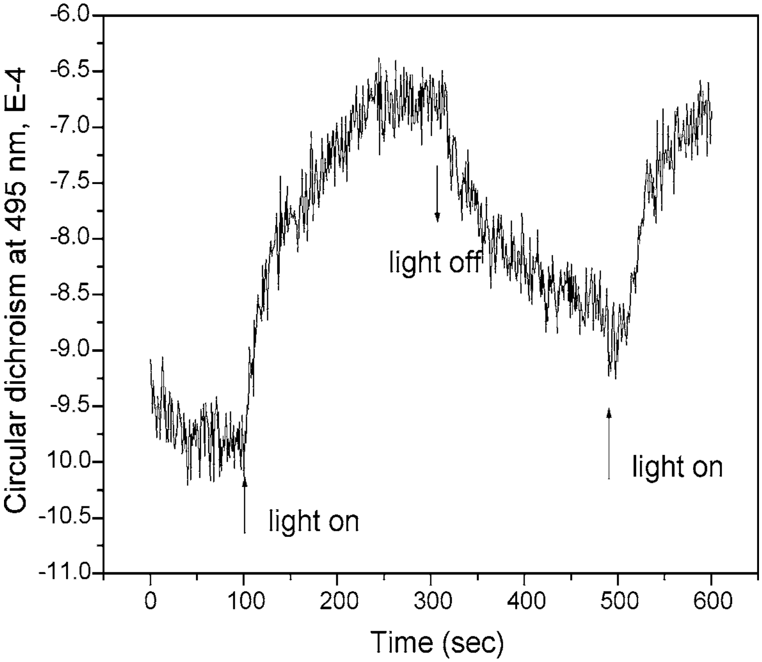

Fig. 2. Light-induced CD changes in Type II LHCII.

This is more convenient than in the red region, where fluorescence transients might contribute to the signal. (In the red, the two filters are interchanged.)

Because ΔCD is sensitive to the temperature, similar to ΔCD in thylakoid membranes ***(19)***, it is essential that the light-induced changes be recorded in the optimum temperature range, between 20 and 30°C. Note that ΔCD in some preparations exhibits opposite sign. This is difficult to predict and not well understood. It seems that in all cases the changes are between two states. This bistability may explain variations in the sign, which does not seem to affect the most commonly studied parameters, such as temperature and light-intensity dependencies, trimer-to-monomer transitions.

4. Notes

1. When using either pea, grown in the greenhouse, or spinach, from the local market or greenhouse, be sure that the plants are not treated with any kind of chemical agents (e.g., insecticides, pesticides). The leaves must be fresh, with good turgor. This is important because the following steps will depend primarily on the plant material chosen. Homogenization of 50–60 g pea needs about 150–200 mL of buffer A and for 400g of spinach use about 300–400 mL buffer A.
2. When discarding the supernatant, make sure the light upper part of the supernatant is also thrown away. Just keep the tube upside-down for a minute until all unnecessary material drips out.
3. Homogenize the pellet until no particles are visible on the wall of Potter homogenizer. The chlorophyll concentration (i.e., the optical density) must be properly adjusted; otherwise there is a high risk that Mg ions cannot precipitate the LHCII.
4. The optimum concentration of Triton X-100 can vary from batch to batch. For example, the upper limit of 0.9% will be used when the material is fresh spinach. The same is valid also for preparation of LHCII microcrystals; when preparing microcrystals keep in mind that addition of higher than 1.6% Triton leads to inability to aggregate LHCII at all.
5. Clouds are visible for preparation of LHCII Types I, II, and partially III. When LHCII Type IV is prepared, no clouds are visible in a suspension and the color is dark-green reddish. For Type IV, if clouds are still present after initial addition of Triton X-100, add some more detergent and stir for a few minutes until clouds disappear.
6. When lamellar aggregates of LHCII are prepared a bigger pellet is obtained and small pellets or just a trace are observed for microcrystals of LHCII.
7. Take only the supernatant; especially with lower concentrations of Triton X-100, there is loose pellet, which can spoil the experiment because it contains some unsolubilized thylakoid membranes and thus many additional proteins; and also a larger amount of lipids.
8. Add Mg^{2+} and K^+ in this order, quickly one after the other. When this order is changed no aggregation is possible and the whole experiment might fail.

9. Addition of Triton X-100 should not exceed 0.2% final volume and shaking the tube with the detergent has to be short, 2–3 min followed by the addition of Mg^{2+} and K^+ in this order; shaking continues for another 10–15 min, stop when clouds are no longer generated. Addition of the detergent has to be adjusted after measuring the optical density—higher detergent concentration for higher optical density.

5. Note Added in Proof

Recently the nature and sequence of thermo-optically inducible changes have been identified in granal thylakoid membranes: light induces (i) unstacking of membranes, followed by (ii) a lateral disassembly of the chiral macrodomains, and (iii) monomerization of the LHCII trimers ***(24)***.

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13

Separation, Purification, and Characterization of Polypeptide Composition of Subcomplexes of the Main Light-Harvesting Chlorophyll *a/b*–Protein Complex of Photosystem II

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Summary

The protocols regarding separation and purification of LHCII subcomplexes by non-denaturing isoelectric focusing (ndIEF), confirmation of trimeric nature of the subcomplexes, and establishing their polypeptide composition are described below.

Key Words: Circular dichroism; denaturing isoelectrofocusing; electrophoresis; immunoblotting; light-harvesting chlorophyll *a/b*–protein complex of photosystem II (LHCII); monomer; non-denaturing isoelectrofocusing; polypeptide; stacked thylakoid membrane; subcomplex; trimer, ultracentrifugation.

1. Introduction

Light interception and funnelling of excitation energy toward the reaction center of photosystem (PS) II is mediated by an array of chlorophyll *a/b*–protein complexes. The most abundant of them, called LHCII, constitutes a complicated supramolecular structure with a few levels of structural heterogeneity. Heterogeneity at the DNA level stems from an occurence of multiple coding genes, constituting members of *Lhcb* extended gene family. Individual genes of *Lhcb1-3* types code for various Lhcb1-3 apoproteins differing with respect to their molecular masses and/or isoelectric point (pI) values ***(1)***, nevertheless arrays of pigment molecules (chlorophylls and xantophylls) associated with individual LHCII apoproteins appear identical with regard to their stoichiometry per single copy of apoprotein and their organization. The heterogeneity at the level of pigment–protein holocomplex stems from the fact, that in mature thylakoid membranes LHCII occurs as a population of nonidentical pigment–protein building blocs (subcomplexes) that based on structural in vitro studies

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are thought to be trimeric *(2,3)*. The subcomplexes have been shown to be homo- or heterotrimeric permutations of individual Lhcb1-3 apoproteins, each one associated with identical array of pigment molecules *(4,5)*.

The goal of this chapter is to present protocols that have been invented and applied to separate, purify, and analyze polypeptide composition of an individual LHCII trimeric subcomplexes. This chapter provides descriptions of complete procedures, including notes on how to overcome possible difficulties, with relation to the following:

1. Separation and purification of LHCII subcomplexes by non-denaturing isoelectric focusing (ndIEF) of stacked thylakoid membranes or LHCII particles, separation of mono- and trimeric forms of LHCII.
2. Running circular dichroism spectra of mono- and trimeric forms of LHCII as well as of LHCII subcomplexes to confirm trimeric nature of the subcomplexes.
3. Analysis of polypeptide composition of LHCII subcomplexes by a combination of electrophoretic, and immunological and denaturing isoelectric focusing approaches.

2. Materials

1. Fresh leaves of carnation (*Dianthus caryophyllus L.*) and *Arabidopsis thaliana L.*
2. Rotating-knife blender.
3. Gauze.
4. Centrifugation and ultracentrifugation equipment.
5. Homogenization buffer: 0.4 *M* NaCl, 20 m*M* Tricine/NaOH, pH 8.0, 2 m*M* $MgCl_2$, 0.2% BSA.
6. Hypotonic buffer: 0.15 *M* NaCl, 20 m*M* Tricine/NaOH, pH 8.0, 5 m*M* $MgCl_2$, 0.2% BSA.
7. MNH buffer: 5 m*M* $MgCl_2$, 15 m*M* NaCl, 20 m*M* Hepes/KOH, pH 7.5.
8. Triton X-100.
9. 2 m*M* EDTA, pH 7.5.
10. Glycerol.
11. MSK buffer: 1 *M* sucrose, 300 m*M* KCl, 40m*M* Mes/NaOH, pH 6.0.
12. *n*-Heptylthioglucoside.
13. TM buffer: 20 m*M* Tris/maleate, pH 7.0.
14. *n*-Dodecyl-β-D-maltoside.
15. Acrylamide, *N, N'*-methylene-bisacrylamide, ampholine carrier ampholites, riboflavine.
16. SDS-PAGE equipment.
17. 20 m*M* NaOH.
18. 20 m*M* H_3PO_4.
19. pI marker proteins.
20. 2 m*M* Tris/maleate, pH 7.0.
21. Electroelution equipment.
22. Lithium dodecyl sulphate.

23. Octyl β-D-glucopyranoside.
24. Dichrograph.
25. Electrophoresis 6 × concentrated loading buffer: 375 m*M* Tris-HCl, pH 6.8, 12% sodium dodecyl sulphate, 60% glycerol.
26. Trichloroacetic acid.
27. CBB G-250/1 staining solution: 0.08% Coomassie Brilliant Blue G-250, 0.8% H_3PO_4, 8% $(NH_4)_2SO_4$, 20% methanol.
28. Molecular weight marker proteins.
29. Methanol.
30. Transfer buffer: 25 m*M* Tris, pH 8.3, 192 m*M* glycine, 0.1% sodium dodecyl sulphate, 20% methanol.
31. Nitrocellulose filter, foam sheets, filter paper sheets.
32. Electrotransfer equipment.
33. TBST buffer: 2.5 m*M* Tris-HCl, pH 7.6, 0.8% NaCl, 0.05% Tween-20.
34. TBS buffer: 2.5 m*M* Tris-HCl pH 7.6, 0.8% NaCl.
35. Anti *Lhcb* 1, 2, and 3 primary antibody.
36. Biotin/species-specific secondary antibody conjugate.
37. Streptavidine/horseradish peroxidase conjugate.
38. DAB staining solution: 1.15% Na_2HPO_4, pH 7.4, 0.34% NaH_2PO_4, 0.58% NaCl, 0.03% $NiCl_2$ 0.015% H_2O_2.
39. Acetone.
40. Urea.
41. Isoelectrofocusing equipment.
42. CBB G-250/2 staining solution: 0.08% CBB G-250, 0.8N H_2SO_4, 0.09N KOH, 12% trichloroacetic acid.

3. Methods

3.1. Isolation of Stacked Thylakoid Membranes (BBY Preparations)

The procedure described below is based on the method of Berthold et al. *(6)* with the modifications described *(7,8)*. Stacked thylakoid membranes are produced by detergent (Triton X-100) fragmentation of thylakoids, isolated by osmotic rupture of chloroplasts. Low amounts of Triton X-100 cause a selective breakage at unstacked (grana margins, end membranes, stroma lamellae) thylakoid regions while leaving stacked thylakoid membranes intact (**Fig. 1**). High-salt conditions are administered throughout the procedure starting from the osmotic rupture of chloroplast stage (see the composition of hypotonic and MNH buffers) to keep stacked thylakoid membranes in an insoluble form (inaccesible to detergent). Broken and intact membrane particles are separated by differential centrifugation, according to variations in size and density.

Stacked thylakoid membranes isolated by the procedure described below represent flat membrane fragments in inside-out orientation, having no tendency to form closed vesicles *(7)*.

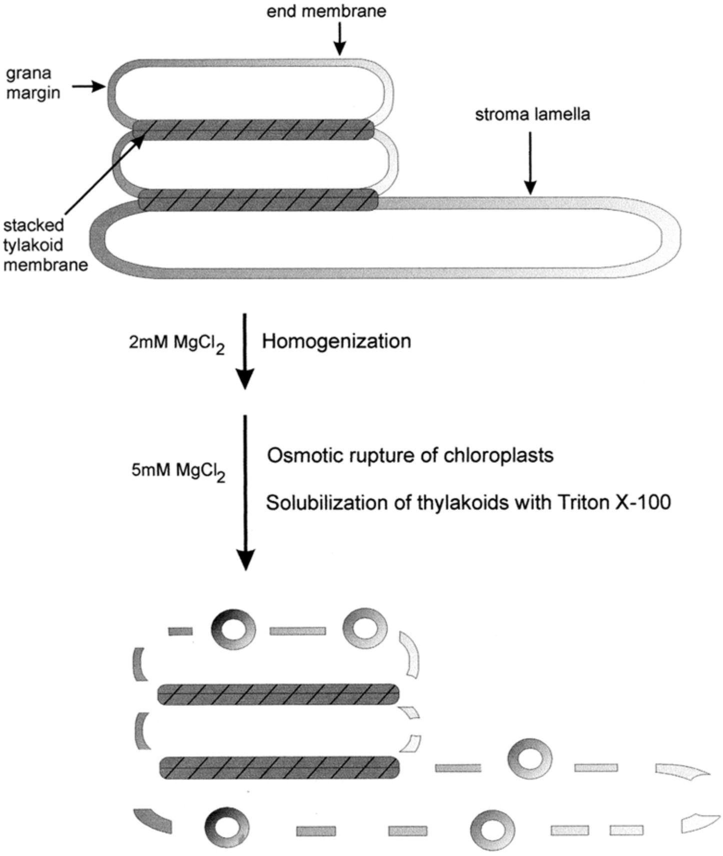


Fig. 1. Triton X-100-dependent fragmentation of thylakoids. Under high-salt conditions Triton X-100 causes a selective rupture of thylakoids at unstacked regions while stacked thylakoid membranes are left intact as flat, inside-out membrane fragments. Small fragments of unstacked thylakoid membranes have a tendency to reseal into right side-out vesicles.

1. Homogenize carnation (*Dianthus caryophyllus L.*) or *Arabidopsis* (*Arabidopsis thaliana L.*) leaves (2 × 20 s) in the homogenization buffer, in a rotating-knife blendor and filter the homogenate through four layers of gauze.
2. Centrifuge the filtrate for 10 min at 4000*g* at 4°C. The pellet now contains about 50% intact chloroplasts.
3. Resuspend the pellet in a hypotonic buffer (osmotic shock medium) and collect the thylakoids by centrifugation for 10 min at 4000*g* at 4°C.
4. Make the thylakoids in a small volume of MNH buffer. Add Triton X-100 to a final concentration of 2.9% (carnation) or 1% (*Arabidopsis*). Keep the final chlorophyll concentration at 2 mg/mL. Solubilize the thylakoids by stirring the mixture for 25 min in the dark at 0°C (*see* **Note 1**).

5. Centrifuge the mixture for 10 min at 3000*g* at 4°C, discard starch-containing pellet and spin the supernatant for 30 min at 40,000*g* at 4°C. Extensively wash the pellet (stacked thylakoid membranes) twice with 2 mM EDTA, pH 7.5 (centrifuging washed pellets for 30 min at 40,000*g* at 4°C), suspend it finally in a small volume of 10% glycerol and store at –20°C. Alternatively, use stacked thylakoid membrane preparation directly for the isolation of LHCII (without suspending it in 10% glycerol).

3.2. Isolation of LHCII

The procedure described below is based on the method of Enami et al. ***(8)*** with modifications described by Jackowski and Kluck ***(9)***. LHCII is isolated by high K^+ concentration (300 m*M* KCl)—induced aggregation from stacked thylakoid membranes solubilized by a detergent (*n*-heptylthioglucoside). LHCII is the only PSII building bloc able to aggregate in vitro in cation-dependent manner.

This feature is believed to be related to te following:

1. LHCII capacity to control thylakoid stacking in vivo by locally decreasing the repulsion of approaching, negatively charged thylakoids in the presence of good electrostatic screening of cations ***(10).***
2. LHCII capacity to promote interthylakoid van der Waals attraction force in vivo ***(11)***.

Aggregated LHCII and nonaggregated fragments of stacked thylakoid membranes are separated by differential centrifugation.

1. Suspend the pellet of stacked thylakoid membrane preparation in the MSK buffer so that the suspension contains 2 mg chlorophyll per mL. Add *n*-heptylthioglucoside to a final concentration of 2.2% and solubilize the membranes by incubating them (with occasional stirring) for 10 min in the dark at 0°C.
2. Introduce 1.5 vol. of MS buffer, keep the suspension for 5 min at 0°C.
3. Centrifuge the suspension at 35,000*g* for 10 min at 4°C, discard the supernatant and extensively wash LHCII-containing pellet twice with 2 m*M* EDTA, pH 7.5 (centrifuging washed pellets for 5 min at 40,000*g* at 4°C), suspend it finally in a very small volume of 10% glycerol and store at –20°C.

3.3. Separation and Purification of LHCII Subcomplexes by Non-Denaturing Isoelectric Focusing of Stacked Thylakoid Membranes or LHCII

Outlined below are the steps utilized to yield LHCII subcomplexes in pure form by the application of ndIEF system in vertical polyacrylamide slab gels, a mild technique utilizing pI as resolving parameter ***(12)***.

1. Add *n*-dodecyl-β-D-maltoside to the preparation of stacked thylakoid membranes or LHCII to the final concentration of 1% (LHCII) or 2% (stacked thylakoid membranes).

2. Fix the final chlorophyll concentration as 1–3 mg/mL (LHCII) or 1.5 mg/mL (stacked thylakoid membranes).
3. Solubilize the preparations by incubating them (with occasional stirring) for 25 min in the dark at 0°C. Spin the surfactant extract at 12,300*g* for 5 min at 4°C to remove insoluble residue.
4. Apply solubilized sample of stacked thylakoid membranes or LHCII to a well of precooled vertical slab gel containing 7% acrylamide, 0.19% *N,N*′-methylene-bis-acrylamide, 2% ampholine carrier ampholites (pH 3.5–5.0), *n*-dodecyl-β-D-maltoside (0.47% in the case of the resolution of stacked thylakoid membranes and 0.375% in the case of LHCII) and 0.00075% riboflavine as polymerizing agent using SDS-PAGE equipment. Use 0.5 mm (analytical purposes) or 1.5 mm (preparative purposes) -thick gel. Apply 20 m*M* NaOH as the cathode solution and 20 m*M* H_3PO_4 as the anode solution. Take special care when overlayering samples applied to the wells of the gels with cathode solution. The samples form a flat layer at the bottom of the well and may not be mixed with cathode solution.
5. Focus the samples under continuous cooling for a total of 5–6 h at constantly increasing voltage (50 –250V in 50V/30 min increments and 300–500V in 100V/1 h increments). Protect the gel from light with an aluminium foil cover during the run.
6. Electroelute the green bands from the gel using TM as an electroelution buffer. Add *n*-dodecyl-β-D-maltoside and glycerol to the eluates to yield 1% (2%) and 10% final concentrations, respectively, solubilize the samples for 25 min on ice and purify by rerunning on a second IEF gel followed by second electroelution. Determine pH of resulting green bands with respect to the series of pI marker proteins (**Fig. 2**) (*see* **Note 2–5**).

3.4. Separation of Monomeric and Trimeric Forms of LHCII

Monomeric and trimeric forms of LHCII may be separated by a nonequilibrium, rate-zonal ultracentrifugation of solubilized LHCII sample in sucrose gradient (13) owing to the large differences in size and shape.

1. Centrifuge LHCII preparation for 5 min at 40,000*g* at 4°C.
2. Suspend LHCII pellet in 2 m*M* Tris/maleate, pH 7.0, so that the suspension contains 1.2 mg chlorophyll per mL. Add lithium dodecyl sulphate, octyl β-D-glucopyranoside and *n*-dodecyl-β-D-maltoside to yield final concentrations of 1.2, 2.4, and 1.2%, respectively (carnation) or 0.2, 0.4, and 0.2%, respectively (*Arabidopsis*). Solubilize LHCII by incubating the suspension for 30 min on ice. Spin the surfactant extract for 5 min at 12,300*g* at 4°C to remove insoluble residue.
3. Load solubilized LHCII on top of 0.2–0.8 *M* sucrose gradient containing 2 m*M* Tris/maleate, pH 7.0, and run at 300,000*g* for 6.5 h at 4°C (**Fig. 3**). Harvest monomeric (upper green band) and trimeric (lower green band) forms of LHCII with a syringe.

3.5. Running Circular Dichroism Spectra of Mono- and Trimeric Forms of LHCII and of LHCII Subcomplexes

Circular dichroism spectra of monomeric and trimeric forms of LHCII vary with respect to chl Q_y as well as Soret region *(14,15)*. Recording room temper-

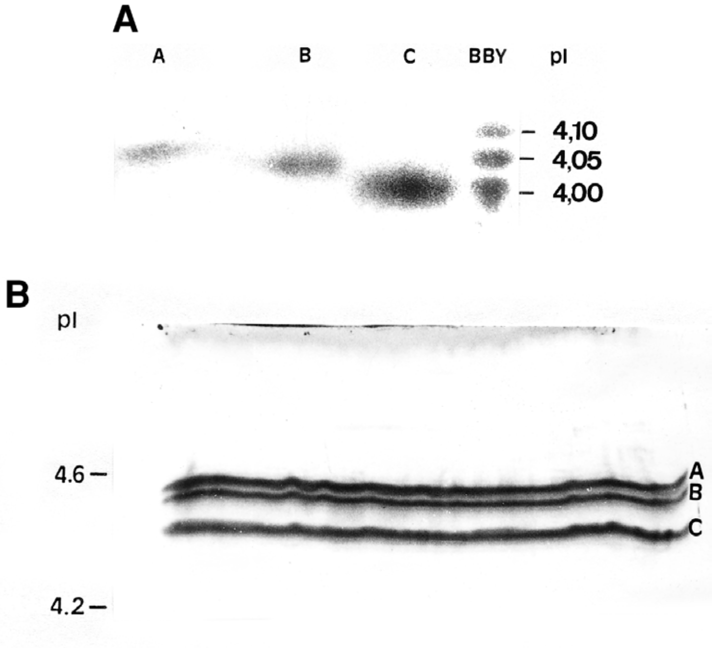


Fig. 2. Fractionation of stacked thylakoid membranes (BBY preparations) and LHCII by non-denaturing isoelectrofocusing. 0.03 mg chlorophyll of BBY preparation isolated from *Arabidopsis thaliana* chloroplasts and 0.02 mg chlorophyll of LHCII subcomplexes labelled **A–C**, electroeluted from the gel after preparative non-denaturing isoelectrofocusing, were solubilized with 2%. *n*-Dodecyl-β-D-maltoside and resolved in a 7% polyacrylamide slab gel containing 0.47% *n*-dodecyl-β-D-maltoside **(A)**. 0.5 mg chlorophyll of LHCII sample isolated from carnation chloroplasts were solubilized in 1% *n*-dodecyl-β-D-maltoside and resolved in a 7% polyacrylamide gel containing 0.75% *n*-dodecyl-β-D-maltoside. Individual LHCII subcomplexes were labeled A, B, and C **(B)**. pI values of pI marker proteins are shown to the right (A) or left (B). The gels were not stained. In lane BBY in (A) only LHCII containing fragment of the gel is shown. (Reproduced from refs. ***5*** **[A]** and ***12*** **[B]** with permission.)

ature circular-dichroism spectra of monomeric and trimeric LHCII forms separated as described in **Subheading 3.4.**, allows the demonstration of two excitonic features that have more enhanced amplitude in trimer than in monomer of LHCII, namely 414 nm (+) and 493 nm (–) *(5)*. The 493 nm (+) feature was described earlier as "quenching" chlorophyll *b* exciton, gaining in strength in aggregated trimers with respect to monomers of LHCII. Both excitonic features appear significant in circular dichroism spectra of LHCII subcomplexes isolated and purified by ndIEF confirming this way trimeric status of the subcomplexes *(5)*.

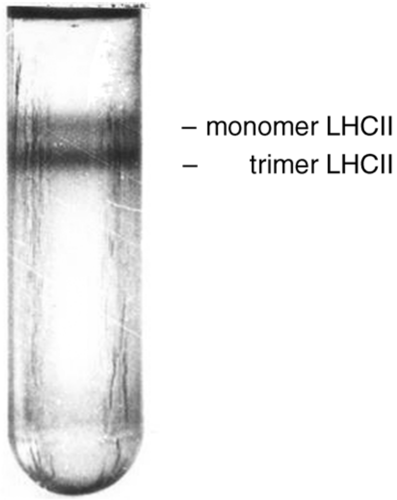


Fig. 3. Resolution of LHCII by ultracentrifugation in a sucrose gradient. 0.07 mg chlorophyll of LHCII sample isolated from carnation chloroplasts were solubilized with 1.2% lithium dodecyl sulphate, 2.4% octyl β-D-glucopyranoside and 1.2% *n*-dodecyl-β-D-maltoside and spun at 300,000*g* in 0.2–0.8 *M* sucrose gradient.

Run room temperature circular dichroism spectra (400–700 nm) of monomeric and trimeric forms of LHCII harvested from sucrose gradients as well as LHCII subcomplexes electroeluted from ndIEF gel (0.005 mg chlorophyll per mL) using a dichrograph (**Fig. 4**).

3.6. Analysis of Polypeptide Composition of LHCII Subcomplexes

Outlined below are the electophoretic, dIEF-based, and immunological approaches that may be applied to examine polypeptide compositon of LHCII trimeric subcomplexes in terms of permutations of various *Lhcb 1-3* gene products, their molecular masses and subsets of pI isoforms. Electrophoretic procedure is SDS-PAGE applying buffer system of Laemmli ***(16)***, dIEF procedure is based on the protocol described by ***(17)*** and the immunological analysis is performed according to the method described by Jansson et al. ***(18)***. Staining the gels after SDS-PAGE is based on a protocol published by Neuhoff et al. ***(19)*** and staining the ones after dIEF is performed according to the method described by Blakesley and Boezi ***(20)***.

1. Mix electroeluates representing individual LHCII subcomplexes with concentrated electrophoresis loading buffer, heat them for 2 min at 100°C, cool rapidly on ice, apply to the wells of 14% acrylamide slab resolving gel and run the gel at 25 mA for 3 h using SDS-PAGE equipment.

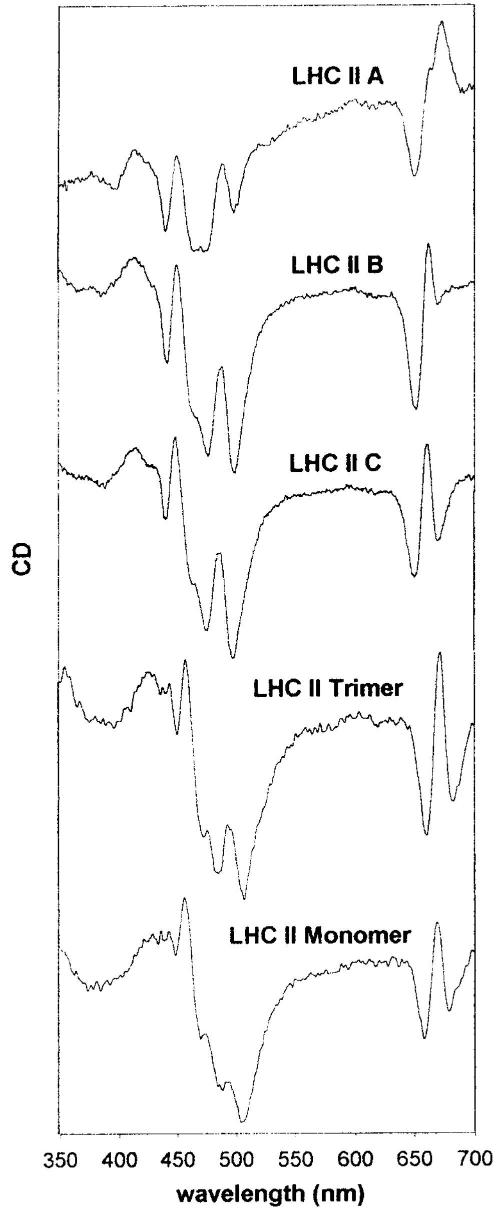
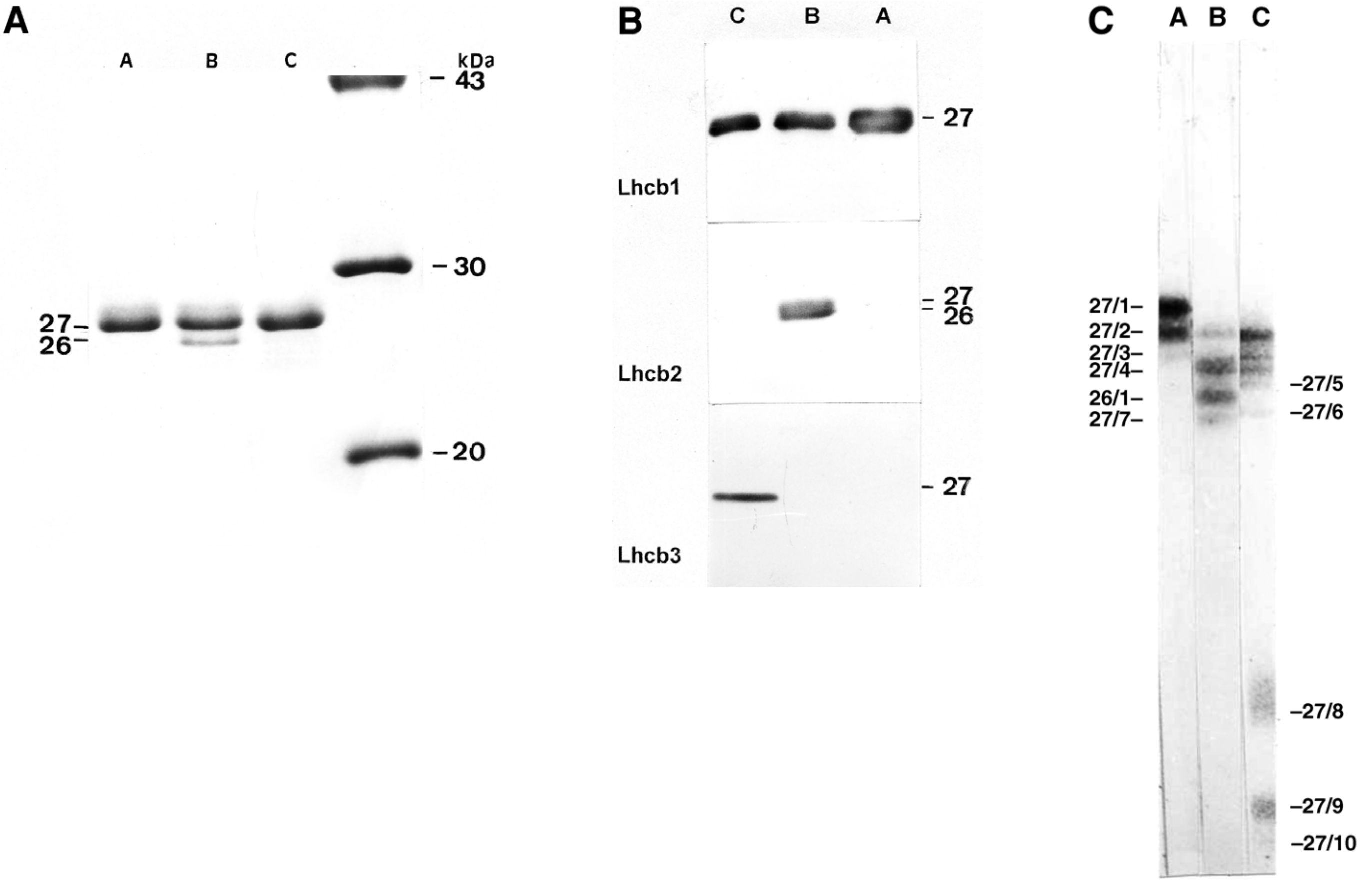


Fig. 4. Circular dichroism spectra of LHCII subcomplexes as well as mono- and trimeric forms of LHCII. Circular dichroism spectra were run for LHCII subcomplexes, labeled A–C, isolated and purified from *Arabidopsis thaliana* chloroplasts by two consecutive runs of non-denaturing isoelectrofocusing and for mono- and trimeric forms of LHCII isolated and purified from *Arabidopsis thaliana* chloroplasts by ultracentrifugation in sucrose gradient. (Reproduced from ref. **5** with permission.)

2. Fix the gel for 30 min in 12% trichloroacetic acid.
3. Stain the gel overnight with CBB G-250/1 solution and destain it in 20% methanol as long as necessary to achieve satisfactory visualization of the bands (usually several hours) (**Fig. 5A**). Determine molecular weight of resulting blue bands with respect to the series of molecular weight marker proteins.
4. After an electrophoretic separation of polypeptides of LHCII subcomplexes rinse all the components of the electrotransfer sandwich (i.e., the gel, nitrocellulose filter, foam sheets, and filter paper sheets) in transfer buffer for 30 min under constant shaking.
5. Assemble the electrotransfer sandwich, and transfer the polypeptides on the nitrocellulose filter for 12–16 h at 30V at 4°C using transfer buffer and standard electrotransfer equipment.
6. Disassemble the sandwich, rinse nitrocellulose filter for 10 min at room temperature in transfer buffer under continuous shaking. Wash the filter in TBST buffer under the conditions described for rinsing nitrocellulose. All remaining steps are performed at room temperature under constant shaking.
7. Wash the filter twice with water.
8. Incubate the filter in TBS buffer containing 3% bovine serum albumine for 1 h.
9. Wash the filter with TBST buffer (2 × 10 min + 1 × 15 min).
10. Incubate the filter with primary antibody (anti - Lhcb 1, 2, or 3) dissolved in TBST buffer containing 1% bovine serum albumine for 1.5 h.
11. Wash the filter in TBST buffer (4 × 6 min).
12. Incubate the filter with biotin-conjugated species-specific secondary antibody dissolved in TBST buffer containing 1% bovine serum albumine for 1 h.
13. Wash the filter in TBST buffer (3 × 10 min).
14. Incubate the filter with streptavidine/peroxidase conjugate dissolved in TBST buffer containing 1% bovine serum albumine for 1 h.
15. Wash the filter in TBST buffer (3 × 10 min).
16. Stain the filter with DAB staining solution under dim light for several minutes.
17. Destain the filter with water (2 × 1 min) and dry it (**Fig. 5B**).
18. Precipitate polypeptides of LHCII subcomplexes by adding 5 volumes of an ice-cold 80% acetone to the electroeluates. Keep the suspenison for 24 h at –18°C.

Fig. 5. (*Opposite page*) SDS-PAGE, immunoblot and denaturing isoelectrofocusing analysis of polypeptide compositon of LHCII subcomplexes. 0.02 mg chlorophyll of polypeptides of LHCII subcomplexes labeled A–C isolated and purified from carnation chloroplasts by non-denaturing isoelectrofocusing were resolved by SDS-PAGE **(A)**, transferred onto nitrocellulose filter and immunostained with anti Lhcb1-3 antibodies **(B)** or resolved by denaturing iselectrofocusing **(C)**. The gels in A and C were stained with Coomassie Brilliant Blue. Apparent molecular weights of protein markers are shown to the right (A,B). In lane (C) the positions of pI isoforms of 27 kDa (27/1-10) and 26 kDa (26/1) polypeptides are shown. (Reproduced from refs. ***12*** [A and C] and ***4*** [B] with permission.)


A
A
B
C
kDa
43
30
20
27
26
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C
B
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27
Lhcb3
C
A
B
C
27/1
27/2
27/3
27/4
26/1
27/7
27/5
27/6
27/8
27/9
27/10

19. Collect the pellets by centrifuging the suspension at 12,300*g* for 10 min at + 4°C. Wash the pellets once with an ice-cold 100% acetone and centrifuge at 12,300*g* for 10 min at +4°C. Dissolve the pellets in a isoelectrofocusing loading buffer.
20. Prefocus rod gel containing 5.5% acrylamide, 0.15% *N,N'*-methylene-bisacrylamide, 2% Triton X-100, 10% glycerol, 8 *M* urea, 2% ampholine carrier ampholytes (3.5–5.0 pH, 4.0–6.0 pH and 3.5–10 pH blended at 1: 5.7 : 1.7 stoichiometry) and 0.0015% riboflavine as a polymerizing agent. under continuous cooling for a total of 75 min at increasing voltage (50 V; 15 min, 100 V; 30 min, 150 V; 30 min). Use 20 m*M* NaOH as the cathode solution and 20 m*M* H_3PO_4 as the anode solution.
21. Apply the samples of polypeptides of LHCII subcomplexes to the gel rods and focus them at constantly increasing voltage (50–500V in 50V; 30-min increments) using the same electrode solutions as the ones administered during prefocusing. Stain the gels rods overnight with CBB G-250/2 solution and destain them in water as long as necessary to achieve satisfactory visualization of the bands (usually several minutes) (**Fig. 5C**).

4. Notes

1. When using leaves of plant species other than *Dianthus catophyllus L.* or *Arabidopsis thaliana L.* final Triton X-100 concentration required to get pure BBY preparation shall be established empirically for each individual species. The concentration selected shall yield the BBY samples of lowest chlorophyll *a/b* ratio possible.
2. One of the commonest problem with this method is the appearance of the disturbances in the pH gradient resulting in distorted bands. pH gradient may be disturbed due to excessive amount of salt in the sample or insufficient cooling of the gel. When the problem arises it is recommended to perform additional washings of the sample with 2 m*M* EDTA pH 7.5 before application to the gel (when the problem is induced by excessive salt amount) or make sure there is a good contact between cooling plate and undersurface of the gel (when the problem is induced by insufficient cooling).
3. Another difficulty that may occur is sample aggregation within the wells of the gel before starting the resolution, resulting in incomplete penetration of the gel by the sample. To overcome this problem it is recommended to:
    a. Perform additional washings of the sample with 2 m*M* EDTA, pH 7.5, (as the aggregation may occur owing to excess of salt in the sample).
    b. Always use freshly prepared solutions of *n*-dodecyl-β-D-maltoside as the solubilizing agent making sure a correct acrylamide concentration within the gel was used (7%).
4. Lack of sample resolution during the run may be overcome by using adequate acrylamide concentration and freshly prepared solution of *n*-dodecyl-β-D-maltoside as the solubilizing agent.
5. When all potential problems are overcome LHCII subcomplexes are separated by the method with the resolving power of between 0.01 and 0.02 of a pH unit.

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14

Isolation of CP43 and CP47 Photosystem II Proximal Antenna Complexes From Plants

Rafael Picorel, Miguel Alfonso, and Michael Seibert

Summary

A single-column method to purify the CP43 and CP47 pigment–protein complexes of photosystem (PS)II from higher plants is presented. To validate the isolation procedure, three different species were used (*Spinacea oleracea, Beta vulgaris,* and *Glycine max*), and the procedure worked similarly with all three. Oxygen-evolving core complex obtained from highly enriched PSII membrane fragments were used as the starting material. The core complex is treated with the chaotropic agent $LiClO_4$ and the nonionic detergent *n*-dodecyl β-D-maltoside. After dialysis against buffer with no detergent or chaotropic agent, the solubilized material is separated by weak anion-exchange chromatography using a TSK-GEL Toyopearl DEAE 650s column. CP43 complex does not bind to the column and elutes with the first pigmented fractions. When the eluate becomes colorless, the column is subjected to a 0–175 m*M* $LiClO_4$ linear gradient. The main pigment elution band corresponds to CP47 complex. The last pigmented elution band contains both reaction center-CP47 and reaction center complexes.

Key Words: Anion-exchange chromatography; CP43; CP47; isolation; photosystem II; pigment–protein complexes.

1. Introduction

Photosystem (PS)II is a membrane-bound, pigment–protein complex present in all oxygenic photosynthetic organisms, and it is composed of a large number of protein subunits and cofactors. The PSII core complex, an integral part of PSII, is surrounded by the distal antenna complexes, including LHCII, CP29, CP26, and CP24. It can be isolated from plants, algae, and cyanobacteria, and it retains the ability to perform O_2 evolution. The core complex is composed of the D1-D2-cytochrome (Cyt) b_{559} reaction center complex (the site of primary charge separation), the CP43 and CP47 proximal antenna complexes, and the 33-kDa extrinsic protein. The names of the proximal antenna complexes, CP,

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means chlorophyll (Chl) protein, are derived from their apparent molecular masses as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the single polypeptide present in each complex. CP43 and CP47 are encoded by the chloroplast genes, *psbC* and *psbB*, respectively. According to the three-dimentional structure of the core complex from cyanobacteria ***(1,2)***, CP43 is physically closer to the D1 and CP47 to the D2 reaction center polypeptides. The reason for this is unclear, but CP43 seems to be more affected by photoinhibitory conditions than CP47 ***(3)***, perhaps because D1 is the main target of that process. These pigment–protein complexes probably function as a link for excitation energy transfer between the distal antenna complexes and the reaction center. However, other functions have also been attributed to the proximal antenna, including their implication in the assembly of the active oxygen-evolving complex ***(3)***. The purification of native CP43 and CP47 complexes, and their site-directed mutant analogues, is necessary to assess their physical, chemical, and spectroscopical properties without interferences from other photosynthetic pigmented proteins, to determine their precise chemical composition, and to carry out in vitro reconstitution experiments. In this chapter we describe a purification method to isolate CP43 and CP47 complexes using a single anion-exchange chromatography column. The column is used to process material obtained from the solubilization of a high-quality core complex using detergent in the presence of a chaotropic agent. This method has been used successfully with core complexes from three different plants.

2. Materials

1. PSII core complexes from *Spinacea oleracea, Beta vulgaris,* and *Glycine max* (*see* **Note 1** and Chapter 4).
2. TSK-GEL Toyopearl (Fractogel in North America) DEAE 650s anion-exchange resin.
3. *n*-Dodecyl β-D-maltoside (DM).
4. Liquid chromatography column, a fraction collector and chromatogram plotter.
5. A high-performance liquid chromatography apparatus (HPLC).
6. A reversed-phase liquid chromatography column.
7. Buffer 1: 0.8 *M* Tris-HCl, pH 8.5.
8. Buffer 2: 50 m*M* MES, pH 6.0, 200 m*M* NaCl, and 300 m*M* sucrose.
9. Buffer 3: 50 m*M* MES, pH 6.0, 400 m*M* sucrose, and 10 m*M* NaCl.
10. Buffer 4: 20 m*M* BIS-Tris-HCl, pH 6.0, 4 *M* $LiClO_4$, and 15% DM.
11. Buffer 5: 20 m*M* BIS-Tris-HCl, pH 6.0.
12. Buffer 6: 20 m*M* BIS-Tris HCl, pH 6.0, 0.05% DM.

3. Methods

The basic protocols of this method have been published in Alfonso et al. ***(4)***, but they are somewhat modified in the following sections. All procedure were carried out at 4°C in the dark (*see* **Fig. 1** for a flow diagram).

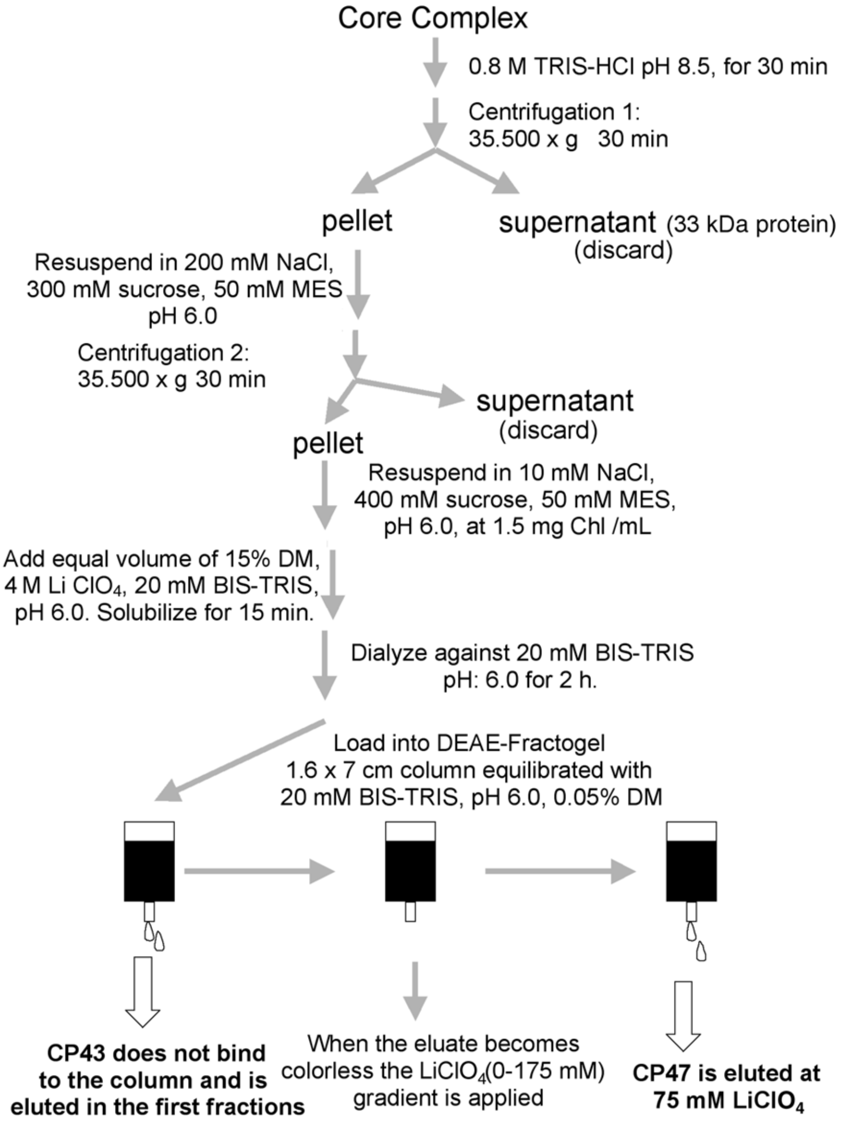

Fig. 1. Flow-chart diagram of CP43 and CP47 purification.

3.1. Solubilization of Core Complexes

1. Incubate high-quality PSII core complexes (*see* **Note 1** and Chapter 4) with buffer 1, for 30 min with gentle stirring to remove the 33-kDa extrinsic protein (*see* **Note 2**).
2. Centrifuge the suspension at 35,500*g* for 30 min.
3. Resuspend the pellet in buffer 2.

4. Centrifuge the suspension once more under the same conditions.
5. Resuspend the resultant pellet at 1.5 mg Chl/mL in buffer 3.
6. Mix the suspension with an equal volume of buffer 4, and incubate for 15 min with occasional hand shaking (*see* **Note 3**).
7. Dialyze the solubilized material in 10-kDa molecular cutoff tubing against buffer 5.

3.2. Ionic-Exchange Chromatography

1. Load the dialyzed material onto a TSK-GEL Toyopearl DEAE 650s weak anion-exchange column (1.6 × 7.5 cm), previously equilibrated with buffer 6 at a flow rate of 2 mL/min.
2. Wash the column with the same buffer at a flow rate of 1 mL/min, and collect 0.5 mL fractions of the eluate. The first green-colored elution fractions correspond to CP43 because this complex does not bind to the column under these chromatographic conditions.
3. When the eluate becomes colorless, subject the column to a 0–175 m*M* $LiClO_4$ linear gradient in the same buffer at a flow rate of 0.5 mL/min, and collect 0.5 mL fractions. After the application of the gradient, the first green colored fractions at around 75 m*M* $LiClO_4$ correspond to CP47.
4. Finally, another elution band comes out at around 125 m*M* $LiClO_4$ and it contains CP47-D1/D2/Cyt b_{559} and D1/D2/Cyt b_{559} complexes.

3.3. Room-Temperature Absorption Spectra: Simple and Rapid Selection Criteria for Obtaining High-Quality CP43 and CP47 Preparations

1. Once the chromatography is completed, the room-temperature absorption spectrum of each fraction is taken in the 350–750 nm range, and the spectral characteristics are used as the criteria for selecting the fractions that will constitute pure CP43 and CP47 complex preparations.
2. If necessary, the highly concentrated fractions are diluted with buffer 6 for absorption spectroscopy.
3. The first colored chromatographic fractions exhibit absorption spectra in the red region with a peak maximum at 671 nm and a smooth shoulder at around 682–683 nm. The slope of the red side of the band is less pronounced than that of the blue side. The absorption spectra also show two prominent (around half of the absorption of the 436 nm band) broad bands in the 440–500 nm carotenoid region. Other main peaks are at 436 and 417 nm due to the Soret transitions of Chl *a*. These fractions correspond to CP43.
4. The first strong elution band from the column after application of the $LiClO_4$ gradient (CP47) exhibits absorption spectra with a maximum at 675 nm in the red region; two broad, weak bands in the carotenoid region; and Chl *a* peaks at 436 and 417 nm in the Soret region. The absorption in the carotenoid region is smaller in CP47 than in CP43. In the case of CP43, the absorption of the carotenoid bands is greater than that of the Chl *a* vibronic band at around 620 nm. In CP47 the carotenoid absorption is almost the same as the Chl *a* vibronic band (*see* **Note 4**).

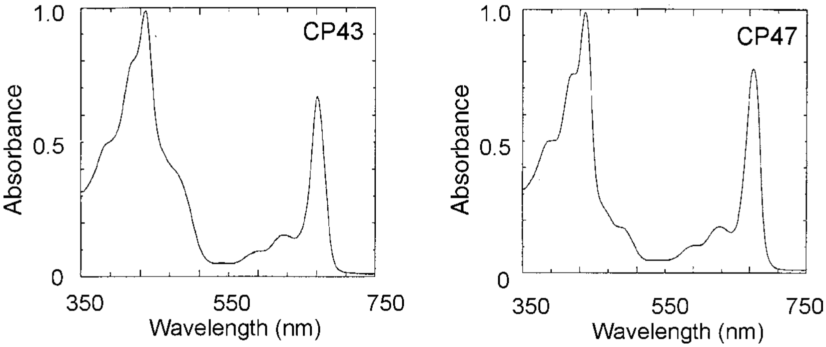

Fig. 2. Room-temperature absorption spectra of isolated CP43 and CP47.

5. A summary of the criteria for simple, rapid selection of the acceptable elution fractions is as follows (*see* **Note 5**):

   CP43: a peak maximum at 671 nm, a smooth shoulder at 682–683 nm, and much higher absorption in the carotenoid region than in the Chl *a* vibronic band at around 620 nm **(Fig. 2)**.

   CP47: a peak maximum at 675 nm and low absorption in the carotenoid region similar to the absorption of the Chl *a* vibronic band at around 620 nm. To increase the quality of the CP43 and CP47 preparations, discard the less concentrated fractions from both sides of the elution bands. The fractions, which constitute the central part of the bands and comply with the above-described criteria, are pooled and dialyzed against buffer 6 for 1 h to eliminate most of the $LiClO_4$ before storing at –80°C until use **(Fig. 2)**.

3.4. Additional Procedures for Analyzing the Quality of the Preparations: Slower But Quantitative Techniques

3.4.1. Low-Temperature Absorption Spectra (see **Note 6**)

Low-temperature absorption spectra of CP43 resolve several bands within the main room-temperature absorption band *(5,6)* peaking at 671 nm. A well-defined, sharp band is observed at 4K at 683 nm, a clearly defined shoulder is apparent at around 678 nm, and the more intense main band appears at 671 nm. In the carotenoid region, two distinct peaks at 495 and 466 nm are well resolved *(4)*. Low-temperature absorption spectra of CP47 in the red region exhibit complex structural features with several bands peaking at around 683, 677, 670, and 660 nm *(7)*. Two well-defined peaks at 503 and 468 nm are also apparent in the carotenoid region *(4)*.

3.4.2. Pigment Analysis by HPLC

Pigments from CP43 or CP47 are extracted with 80% (v/v) cold, aqueous acetone by sonicating the complexes for 1 min. This promotes pigment release from the protein-detergent complexes, and the extracts are centrifuged for 5 min in a microfuge. In both cases, the white pellet, containing detergent and denatured protein, is discarded and the pigment-containing supernatant is injected in an HPLC with a C-18, reversed-phase column using a mobile phase B containing acetonitrile:methanol:water:ethyl acetate (7:0.96:0.04:8) *(8)*. The chromatograms are monitored at 450 nm and show only two main peaks that correspond to Chl *a* and β-carotene. The presence of Chl *b* and xantophylls is 8a clear indication of contamination by other PSII antenna complexes.

3.4.3. Polypeptide Content Analysis by SDS-PAGE

The polypeptide content of CP43 and CP47 can be analyzed by SDS-PAGE using a 12.5% acrylamide gel with 4 *M* urea. Coomassie Brilliant Blue stained gels showing a single band at around 43 and 47 kDa indicate pure CP43 and CP47, respectively.

4. Notes

1. High-quality core-complex preparations (*see* Chapter 4) are a requisite for optimal results using the above described, single-column chromatography procedure. Unfortunately, PSII core complex preparations are often contaminated with various amounts of the so-called minor PSII antenna complexes and possibly other materials. One common contaminant is CP29 that elutes just before CP47 from the TSK-GEL Toyopearl DEAE 650s column, but it can be eliminated easily by discarding the first few fractions of the CP47 elution band. Proteins of 22- and 10-kDa molecular weights are also present in some core preparations. These proteins do not bind to the TSK-GEL Toyopearl DEAE 650s column, and thus, co-elute with CP43. Minor contamination of these proteins should not be a problem if the central part of the CP43 elution band is taken from the column. In the case of more serious contamination by these two proteins, several procedures are available to eliminate these contaminants from CP43 preparations *(5,6,9)*.
2. The elimination of the 33-kDa extrinsic protein before solubilization of the core complex as explained in **Subheading 3.1.** is an important step to obtain pure CP47 complex because both proteins co-elute from the column under our experimental conditions.
3. Preparation of buffer 4, containing 4 *M* $LiClO_4$, should be done at room temperature, and the buffer should then be kept at that temperature until use. Low temperatures result in precipitation of $LiClO_4$. After mixing one part of core complex in buffer 3 (at 4°C) and one part of buffer 4 (at room temperature), the suspension is incubated at 4°C for 15 min as explained in **Subheading 3.1.** All buffers containing DM should be prepared freshly before using.
4. When room-temperature absorption spectra of CP43 preparations are taken in the 750–350 nm region as mentioned in **Subheading 3.3.**, it is helpful to examine expanded spectra in the approx 750–600 nm region. The shoulder at 682–683 nm is more apparent under these conditions, and if it is not apparent, the CP43 preparation can be considered poor quality.

5. The yield of this protocol is normally higher for CP43 than CP47 because a small portion of the latter remains bound to D1-D2-Cyt b_{559} complex. This comes off the column with the last colored fractions.
6. The concentration of $LiClO_4$ and other salts should be reduced as much as possible in the samples after isolation if they are to be used for low-temperature optical studies. Salts can interfere with formation of a transparent glass at low-temperature.

Acknowledgments

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15

The Determination and Quantification of Photosynthetic Pigments by Reverse Phase High-Performance Liquid Chromatography, Thin-Layer Chromatography, and Spectrophotometry

Tessa Pocock, Marianna Król, and Norman P. A. Huner

Summary

Chorophylls and carotenoids are functionally important pigment molecules in photosynthetic organisms. Methods for the determination of chlorophylls *a* and *b*, β-carotene, neoxanthin, and the pigments that are involved in photoprotective cycles such as the xanthophylls are discussed. These cycles involve the reversible de-epoxidation of violaxanthin into antheraxanthin and zeaxanthin, as well as the reversible de-epoxidation of lutein-5,6-epoxide into lutein. This chapter describes pigment extraction procedures from higher plants and green algae. Methods for the determination and quantification using high-performance liquid chromatograpy (HPLC) are described as well as methods for the separation and purification of pigments for use as standards using thin-layer chromatography (TLC). In addition, several spectrophotometric methods for the quantification of chlorophylls *a* and *b* are described.

Key Words: Antheraxanthin; β-carotene; neoxanthin; chlorophyll *a*; chlorophyll *b*; high-performance liquid chromatography; lutein epoxide; lutein; photosynthesis; pigment extraction; pigments; thin-layer chromatography; violaxanthinx anthophyll cycle; zeaxanthin.

1. Introduction

Pigments are functionally important molecules in photosynthetic organisms. They not only harvest the light energy necessary for carbon reduction but some serve to protect the organism from excess light. The balance of photosynthetic pigments is dynamic and contributes to the maintenance of photostasis within the cell *(1)*. In chloroplasts, chlorophyll and some carotenoids are lipophilic and occur as pigment–protein complexes within the thylakoid membrane. Therefore, the membrane has to be disrupted and the pigments must be separated from their associated proteins during their extraction. Carotenoids also occur

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within of the chloroplast envelope, particularly violaxanthin *(2)*. The primary light-harvesting pigments are chlorophylls *a* and *b*. Chlorophyll *a* is situated in the light-harvesting complexes (LHC), the core antennae as well as the reaction centers, whereas chlorophyll *b* is only found in the LHCs. An important characteristic of chlorophyll to keep in mind during their extraction is their lability and sensitivity to light, heat, oxygen, and chemical degradation *(3)*. These conditions should be avoided throughout sample preparation to prevent the possible formation of artifacts and to prevent their degradation.

The second class of photosynthetic pigments, the carotenoids, are more numerous than the chlorophylls. Carotenoids are 40 carbon molecules, formed by joining 8-C5 isoprene units and can be classified as carotenes, hydrocarbons, or xanthophylls. Carotenoids exhibit extensive conjugated double bonds, making them susceptible to oxidation and free radical reactions *(4)*. Xanthophylls, which are hydroxyl- and epoxy-substituted carotenes, play a dual role within the chloroplast. First, they function in light-harvesting and energy transfer within the LHCs. Second, some serve to protect the photosynthetic apparatus from the harmful effects of excess light. The xanthophyll cycle is well described in plants and algae. It is a component of nonphotochemical quenching that involves the dissipation of excess light energy as heat *(5)*. Increases in chlorophyll *a* fluorescence quenching have been directly correlated to increases in zeaxanthin and antheraxanthin with concomitant decreases in violaxanthin *(6)* **(Fig. 1A)**. Changes in the abundance of these pigments can be monitored accurately using the extraction and HPLC methods described below. A unique variation of the xanthophyll cycle is observed in the prasinophyte *Mantoniella squamata* where non-photochemical quenching is antheraxanthin-dependent; the second de-epoxidation to zeaxanthin is absent in this species *(8)*. Interestingly, a new xanthophyll cycle has been observed in the parasitic plant *Cuscuta reflexa* that involves the light dependent de-epoxidation of lutein-5,6-epoxide to lutein *(9)* **(Fig. 1B)**. This confirms previous work performed on lutein deficient mutants of *Arabidopsis* (*lut2*) and *Chlamydomonas* (*lor1*) indicating that lutein has a photoprotective function *(5,10)*. The xanthophyll cycle is induced during periods of excess light and can be seen visually in the HPLC chromatograms for *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* **(Fig. 1)**. Carotenoids are very susceptible to degradation and destruction. Therefore, all procedures should be carried out rapidly and exposure to oxygen, light, heat, and acids should be avoided *(11)*.

Methods for the determination and quantification of neoxanthin, lutein, carotenes, chlorophylls *a* and *b*, violaxanthin, antheraxanthin, and zeaxanthin from plants and algae using HPLC are outlined in the following sections. In addition, separation and purification methods for pigments by TLC are described as well as a rapid method for the quantification of chlorophylls by spectrophotometry.

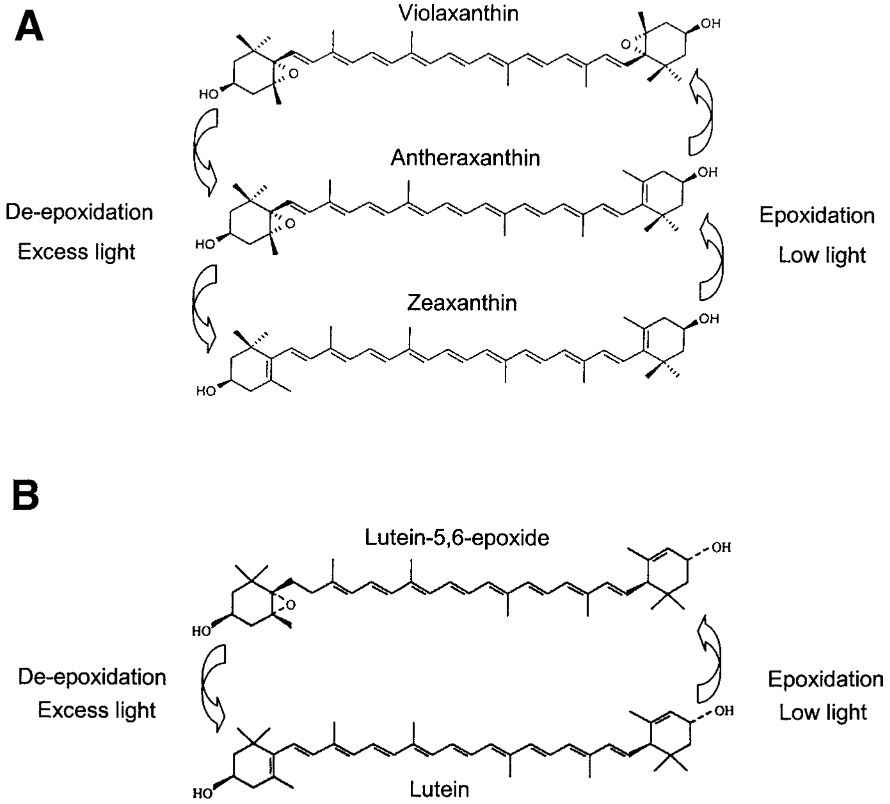

Fig. 1. The light-driven xanthophyll and lutein cycles. (**A**) Reversible conversion of xanthophylls cycle pigments. Under excess light violaxanthin is converted into zeaxanthin through the loss of two epoxy groups resulting in a lengthening of the conjugated double bond system. This conversion can occur on a time scale of minutes whereas epoxidation can occur on a time scale of minutes to hours. (Adapted with permission from ref. *7*.) (**B**) Reversible de-epoxidation of lutein-5,6-epoxide into lutein observed under high light conditions. (Adapted with permission from ref. *9*.)

2. Materials

2.1. High-Performance Liquid Chromatography

1. Beckman System Gold programmable solvent module 126 with diode array detector module 168 (Beckman Instruments, San Ramon, CA). Typical HPLC chromatograms are shown in **Fig. 2**.
2. Beckman 210A sample injection valve with a 20 μL sample loop.
3. Upchurch Perisorb A guard column.

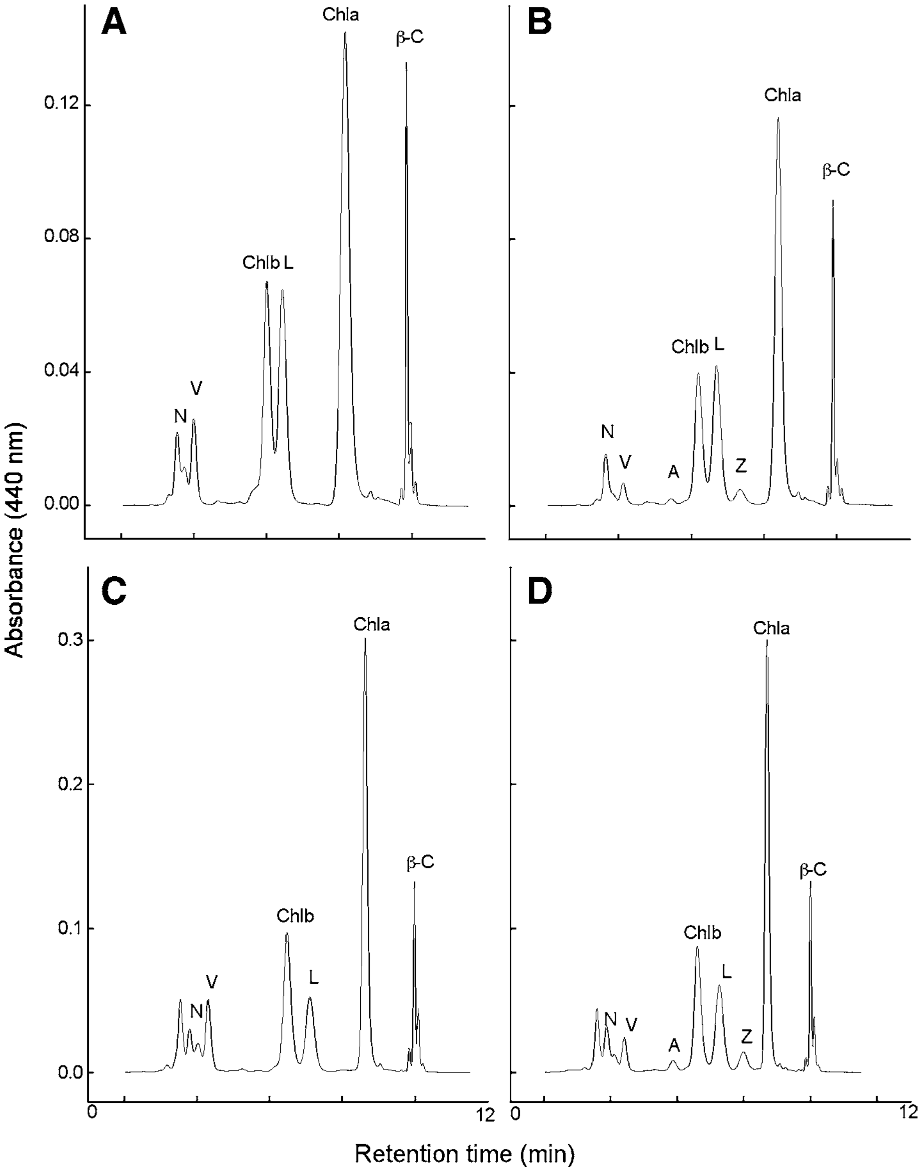

Fig. 2. HPLC chromatograms of *Arabidopsis thaliana* (**A**) and *Chlamydomonas reinhardii* (**C**) before photoinhibition and after a 2 h exposure to high light (**B** and **D** respectively). Note the relative decrease in violaxanthin, and relative increases in antheraxanthin and zeaxanthin in photoinhibited *Arabidopsis* (B) and *Chlamydomonas* (**D**). N, neo-xanthin; V, violaxanthin; Chl*b*, chlorophyll *b*; L, lutein; Chl*a*, chlorophyll *a*; β-C, β-carotene (Pocock, Król, Huner, unpublished).

4. CSC-Spherisorb ODS-1 reverse phase column (5 μm particle size, 25 × 0.46 cm i.d.) (columns from Chromatographic Specialties Inc., Concord, ON, Canada).
5. MINI-BEADBEATER™ (Biospec Products, Inc., Bertlesville, OK).
6. Vacuum flask.
7. 0.45 μm syringe filters and 1 mL syringes.
8. Hamilton syringe.
9. HPLC grade acetone, acetonitrile, methanol, and hexane.
10. Tris[hydroxymethyl]aminomethane, Biotechnology performance certified, Minimum 99.9% (Trizma®Base, Sigma).
11. Zirconia/silica beads 0.1 mm.
12. N_2.
13. 0.1 *M* Tris-HCl buffer (pH 8.0) filtered through a vacuum flask.
14. Solvent A: acetonitrile:methanol:0.1 *M* Tris-HCl (pH 8.0), (72/8/3.5 v/v/v).
15. Solvent B: methanol:hexane (4/1 v/v).

2.2. ThinLayer Chromatography for Purification of Standards

1. Mortar and pestle.
2. Miracloth.
3. Silica Gel 60 TLC plates.
4. Hamilton syringe for loading.
5. Chromatography tank with lid.
6. Filter paper for chromatography tank.
7. Acetone, hexane, chloroform, isopropanol, and ethanol.
8. Petroleum ether (boiling point 60–90°C).
9. Petroleum benzene (boiling point 100–140°C).
10. N_2.
11. $CaCO_3$, $Ca(OH)_2$, MgO.
12. KOH.
13. Petroleum ether:acetone:chloroform (5/5/4 v/v).
14. Petroleum ether:isopropanol:water (100/12/0.25 v/v).
15. Plate preparation medium: $CaCO_3 + Ca(OH)_2 + MgO$ (29.5 g + 5.0 g + 6.0 g) in 60 mL of 1.7% KOH.
16. Separation medium A: Petroleum benzene (boiling point 100–140°C):acetone:chloroform (5/5/4 v/v/v).

2.3. Chlorophyll Extraction

1. Same equipment for pigment extractions as listed previously.
2. Acetone.
3. Sodium phosphate.
4. Buffered acetone for Porra protocol: 80% aqueous acetone containing 2.5 m*M* sodium phosphate buffer (pH 7.8).

3. Methods

3.1. High-Performance Liquid Chromatography

3.1.1. Pigment Extraction From Plants

1. Grind 0.5 g tissue in a chilled mortar and pestle in 100% HPLC grade acetone.
2. Filter extract through Miracloth, centrifuge for 5 min at 3024*g* to clarify.
3. Store at minus –20°C, or preferably –80°C after bubbling with N_2.

3.1.2. Pigment Extraction From Green Alga

1. Centrifuge fresh culture (5 mL) and resuspend the pellet in 1 mL cold 100% HPLC grade acetone at 4°C in dim light.
2. Cells are broken and pigments are extracted in a MINI-BEADBEATER™ (Biospec Products, Inc., Bertlesville, OK) using 0.1 mm zirconia/silica beads for 3 × 20 s cycles.
3. Alternatively, cells can be broken using a French Press. Keep the cell at 4°C until use. Press an aliquot of cell culture (10 mL) twice at 10,000 PSI. Collect samples in a centrifuge tube that is kept cold in a beaker of ice. Place a sample under a light microscope to check for cell disruption.
4. Clarify the supernatant by centrifugation and bubble with N_2.
5. Store at –20°C, or preferably –80°C.

3.1.3. Chromatography

1. Thaw and filter the extracts through 0.45 μm syringe filters.
2. Prime the lines and pumps well.
3. Set the flow rate for 2 cm^3/min.
4. Monitor the baseline until stable.
   Pigments are eluted isocratically for 6 min with solvent A followed by a 2 min linear gradient to 100% solvent B which continues isocratically for 4 min.
5. The total run time is 12 min.
6. Wash the column for 8 min with solvent A before injecting the next sample.
7. Absorbance is detected at 440 nm and peak areas are integrated by Beckman System Gold software.
8. Retention times and response factors must be determined by calibrating the column by injecting known amounts of pure standards.
9. Calculations: To determine the concentration of the pigment of interest the peak area is multiplied by the response factor calculated from the chromatogram for the standard (*see* **Table 1**).

   $$\text{Response factor} = \text{concentration of component/peak area}$$

   To determine the unknown concentration:

   $$\text{Peak area} \times \text{response factor}$$

3.2. Thin-Layer Chromatography

3.2.1. Pigment Extraction for Higher Plants

1. Grind a fresh sample of known weight in a chilled mortar and pestle in 100% acetone at 4°C under green light.
2. Filter sample through Miracloth.
3. Concentrate the supernatant to a small volume by evaporating with N_2.
4. Load a known volume on the TLC plate by spotting sample at the start line with a Hamilton syringe several times while allowing it to dry.

3.2.2. Alternative Method of Pigment Extraction for Higher Plants

If the pigments do not separate well or there is a lot of smearing the following modifications can be applied:

1. The acetone extract is used in phase partitioning with petroleum ether and water saturated with NaCl ***(11)***.
2. Collect the petroleum ether phase.
3. Wash the acetone phase a few times by partitioning in petroleum ether and water.
4. Collect all petroleum ether phases and filter under vacuum through Na_2SO_4 to remove water.
5. Concentrate the pigment solution with N_2 or, if you have a large volume, evaporate it down to 0.5–1 mL in a rotating evaporator keeping the temperature below 35°C with nitrogen in the sample.
6. Load TLC plate as described previously.

3.2.3 Pigment Separation by TLC

This method of TLC plate preparation is good for the separation of zeaxanthin ***(14,15)***.

1. Prepare TLC plates by coating them with the plate preparation medium (*see* **Subheading 2.2.**, **step 15**).
2. Dry the plates at 110°C for 45 min to remove water and keep them in the oven until ready to use.
3. Wrap filter paper around the inside perimeter of the tank.
4. Place the prepared TLC plates in the separation medium A (*see* **Subheading 2.2.**, **step 16**).
5. Cover the tank with the lid and keep in the dark. Separation takes approx 1 h.
6. Dissolve the bands in 1 mL of ethanol (xanthophylls), in hexane (α- and β-carotene) or in acetone (chlorophyll).
7. Calculations: Quantitative determination is performed according to:

$$C= E \times V \times 1000/100 \times G \times E^{1\%}_{1\ \mathrm{cm}}$$

where,

C = concentration in mg carotenoid / g fresh weight

Table 1
The Molecular Weights, Response Factors, Relative Retention Times, and Wavelengths of Maximum Absorbance (λ Max) for Some Carotenoids and Chlorophylls[a]

Pigment	Molecular	Response factor weight	Relative retention time (min)	λ max nm (EtOH)
Neoxanthin	584.00	0.3813	4.046–4.309	415 439 467
Violaxanthin	600.85	0.3813	4.621	419 440 470
Antheraxanthin	584.84	0.4988	6.096	422 444 472
Zeaxanthin	568.85	0.4951	8.642	428 450 478
Lutein	568.85	0.4988	7.538	422 445 474
β-carotene	536.85	0.5530	10.908	448 480 512
				(Acetone)
Chlorophyll *a*	893.48	1.4450	9.292	663
Chlorophyll *b*	907.46	1.3710	6.918	645

The retention times will vary depending on solvents and the column.

E = maximal absorption of a known volume of carotenoid solution read at the wavelength of maximum absorption
V = volume
$E_{1\,\text{cm}}^{1\%}$ = the extinction coefficient of a 1% (w/v) solution in a 1cm cuvet at a defined wavelength (the value of 2500 is used when no specific values are available for an individual carotenoid or for a mixture of pigments)
G = grams of the sample loaded on the TLC plate
The $E_{1\,\text{cm}}^{1\%}$ and wavelength of maximum absorbance (λ max) for individual carotenoids are listed in **Table 2** ***(16)***.

3.3. Chlorophyll Extraction and Determination From Plants

3.3.1. Chlorophyll Extraction From Plants

1. Grind 0.5 g tissue in a chilled mortar and pestle in 5 mL 100% acetone.
2. Filter extract through Miracloth.
3. Centrifuge for 5 min at 3024*g* to clarify.
4. Store covered, on ice until use. If you want to measure chlorophyll later, the sample can be flushed with N_2 and stored at –20°C.

3.3.2. Chlorophyll Analysis Using the Arnon Method

1. 0.05 mL of the pigment extract is added to 0.95 mL of 80% acetone.
2. Absorption is measured at 663 nm (chl a), 645 nm (chl *b*) and 750 nm.
3. Calculations ***(18)***:

Subtract A_{750} from A_{663} and A_{645}
Chlorophyll *a* (mg/g fresh weight) = $(12.7\ A_{663}) - (2.69\ A_{645})$ g fresh weight × V
Chlorophyll *b* (mg/g fresh weight) = $(22.9\ A_{645}) - (4.68\ A_{663})$ g fresh weight × V
Chls *a+b* (mg/g fresh weight) = $(20.08\ A_{645}) + (8.02\ A_{663})$ / g fresh weight × V
where, V = volume of chlorophyll extract (0.05 mL).

3.3.3. Chlorophyll Analysis Using the Porra Method

1. 0.05 mL of the pigment extract is added to 0.95 mL of 80% buffered acetone.
2. Absorption is measured at 663.6 nm (chl *a*) and 646.6 nm (chl *b*).
3. Calculations ***(19)***:

Chlorophyll *a* (μg/mL) = $(12.25\ A_{663.6}) - (2.55\ A_{646.6})$
Chlorophyll *b* (μg/mL) = $(20.31\ A_{646.6}) - (44.91\ A_{663.6})$
Chls *a+b* (μg/mL) = $(17.76\ A_{646.6}) - (7.34\ A_{663.6})$

3.3.4. Chlorophyll Analysis Using the Jeffrey and Humphrey Method

1. Centrifuge an aliquot of fresh culture (5 mL) and resuspend the pellet in 1 mL cold 90% acetone at 4°C in dim light.
2. Break cells in a MINI-BEADBEATER™ as described previously.
3. Clarify the supernatant by centrifugation for 5 min at 3024*g*.
4. Store covered, on ice.
5. Calculations ***(20)***:

Chlorophyll *a* (μg/mL) = $(11.93\ A_{664}) - (1.93\ A_{647})$
Chlorophyll *b* (μg/mL) = $(20.36\ A_{647}) - (5.50\ A_{664})$

4. Notes

1. Solvents must be pure (HPLC grade).
2. Temperature and light: Carotenoid extractions should be performed on ice or at the lowest practical temperature and in diffuse or low light to avoid *cis-trans* (E/Z) isomerization.
3. Oxygen: The oxygen atoms of the hydroxy and epoxy groups of the xanthophylls are derived from molecular oxygen. It has been reported that antheraxanthin exposed to air for 24 h at 50°C was partially oxidized to the diepoxide, violaxanthin ***(13)***. For this reason and to avoid artifacts, carotenoids require careful extraction and subsequent handling. Oxygen is displaced by bubbling the pigment extracts with N_2.
4. Acids: The 5,6-epoxides such as neoxanthin and violaxanthin undergo conversion to the 5,8-epoxide form in the presence of acids. $CaCO_3$ is often added to neutralize any acids present.
5. Response factors, molecular weights, retention times, and wavelength of maximum absorbance (λ max) for a range of pigments are found in **Table 2**.
6. Tris-HCl buffer must be filtered through a vacuum driven filter containing a membrane with pore size of 0.22 μm.

Table 2
Extinction Coefficients and Wavelengths of Maximum Absorbance (λ Max) for Carotenoids and Chlorophylls in Various Solvents

Pigment	Solvent	$E_{1\ \text{cm}}^{1\%}$	λ max
α-Carotene	Hexane	2420	564
β-Carotene	Hexane	2200	464
Lutein-5,6-epoxide	Ethanol	2400	440
Violaxanthin	Ethanol	2500	441
Lutein	Ethanol	2540	446
Antheraxanthin	Ethanol	2350	446
Neoxanthin	Ethanol	2270	438
Zeaxanthin	Ethanol	2480	451
Chlorophyll *a*	Acetone	840	663
Chlorophyll *b*	Acetone	518	645

7. A guard column increases the life of the analytical column. Packing material should be the same material in the analytical column but of larger particle size. When clogged, the filters can be removed, placed in a small amount of solvent used for pigment extraction and sonicated in a sonicating water bath. An alternative is purchasing disposable guard columns. A dirty guard column will increase pressure in the system and compromise the separation while shifting the retention times.
8. If pigment separation is problematic or if retention times shift, increase the column washing with solvent A to 12 min from 8 min between runs.
9. Setting up a new column can be problematic. It is typical to have to test out several columns (even though the packing material is supposedly identical) before selecting one that provides the required resolution to separate the xanthophyll cycle pigments.
10. HPLC chromatograms can be saved as a DIF and opened in Microsoft Excel® after which they can be reproduced in a graphing program such as Sigma Plot® or Origin®.
11. Pure standards of Chl *a*, Chl *b*, lutein and β-carotene were obtained from Sigma, St. Louis, MO.
12. Pure standards of zeaxanthin, violaxanthin, antheraxanthin, and neoxanthin are not available commercially and must be purified by thin-layer chromatography (*see* **Subheading 3.2.**) according to ***(14)*** and Hager and Meyer-Bertenrath ***(15)***.
13. An alternative method for the separation of xanthophylls and carotenes is the modified protocol of Hager and Meyer-Bertenrath ***(15)*** or Król and Huner ***(17)***. Instead of the previously described solvent system, the silica gel 60 plates are placed in either a petroleum ether: isopropanol: H_2O (100/12/0.25) solution or a solution of petroleum ether (boiling point 60–90°C):acetone (70/30).

14. The R_f values are not reproducible but their order of separation is consistent in a particular solvent system. In these separation systems, the order of separation from the top to bottom of the plate is β-carotene, α-carotene, Chlorophyll *a*, Chlorophyll *b*, Lutein, Lutein 5–6 epoxide, Antheraxanthin, Violaxanthin, and Neoxanthin.
15. An unknown pigment can be identified by mixing the sample with a known carotenoid and rechromatographed on TLC plates prepared with MgO-Kieselguhr (1/1, w/w) using the solvent system acetone:petroleum ether (30/70) ***(3)***.
16. Do not use Na_2SO_4 to remove water from the acetone extract before washing and partitioning (**Subheading 3.2.2.**, **step 3** and **4**) as it is difficult to remove pigments from this drying agent.
17. Any equipment, such as chromatography and tanks, should be covered by black cloths or aluminum foil or placed in a dark cupboard.
18. To prevent pheophytin formation, $CaCO_3$ can be added during grinding.
19. The conversion of chlorophyll to pheophytin is prevented by inhibiting chlorophyllase with acetone and in particular buffered acetone.
20. Extinction coefficients are different for various solvents according to **refs. *11*** and ***21***.

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16

Isolation and Identification of Chloroplast Lipids

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Summary

Glycerolipids of photosynthetic organisms are accounted for largely by thylakoid membrane lipids consisting of chloroplast-specific glycolipids, such as monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol, and a sole phospholipid, phosphatidylglycerol. In this chapter, methods for characterization of lipids from plant cells are described. The methods include extraction of total lipids from cells, separation of these lipids into individual lipid classes by thin-layer chromatography (TLC), and identification of respective lipid classes by their mobility. We also show methods for determination of compositions of constituent fatty acids, distribution of fatty acids between *sn*-1 and *sn*-2 positions, and contents of individual lipid classes by gas-liquid chromatography. These methods are applicable to isolated chloroplasts or membrane fractions such as thylakoid membranes.

Key Words: Fatty acid; gas-liquid chromatography; lipase from *Rhizopus delemar*; lipid; methanolysis; thin-layer chromatography.

1. Introduction

Lipids in biomembranes interact among one another through hydrophobic bonds, whereas they interact with membrane proteins not only through hydrophobic bonds, but also with electrostatic and hydrogen bonds. The hydrophobic bonds are eliminated by relatively nonpolar solvents such as chloroform, and the electrostatic and hydrogen bonds are cut by polar solvents such as methanol. Thus, lipids can be prepared from cells or membrane preparations as total lipids through extraction with a mixture of chloroform and methanol. From the total lipid fraction extracted with such a solvent system, individual lipid classes are separated through thin-layer chromatography (TLC) on the basis of their particular mobility, and detected on TLC plates with a nondestructive reagent such as primulin. Fatty acid methyl esters are prepared from individual lipid classes through transmethylation by methanolysis and analyzed

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with gas-liquid chromatography for determination of fatty acid compositions. The chromatography is equipped with a column that separates molecular species of fatty acid methyl esters according to their partition property between gas and liquid phases at high temperatures. Each lipid class can be quantified through estimation of contents of the constituent fatty acids relative to a content of a particular fatty acid that is originally absent, but is added as an internal standard to the lipid fraction. Fatty-acid compositions at the *sn*-1 and *sn*-2 positions of glycerol backbones can also be estimated through determination of fatty acid compositions of both individual lipids and their *sn*-2-acyl-lysolipids synthesized through specific hydrolysis at the *sn*-1 positions by a lipase from *Rhizopus delemar*. Two biosynthetic pathways designated as prokaryotic and eukaryotic pathways operate for glycerolipid synthesis in plants. The prokaryotic pathway, where metabolism is confined within plastids, produces prokaryotic lipids, similar to cyanobacterial lipids, containing fatty acids with a chain of 16 carbon atoms at the *sn*-2 positions. The eukaryotic pathway, through cooperation of three cellular compartments, i.e. plastids, cytoplasm, and endoplasmic reticulum, produces eukaryotic lipids possessing fatty acids with a chain of 18 carbon atoms at the *sn*-2 positions. Thus, information not only on their molecular structure, but also on their synthetic pathway is provided by the analysis of positional distribution of fatty acids of individual lipid classes. It has been shown that chloroplast lipids are synthesized by pokaryotic and/or eukaryotic pathways, although extra-chloroplast lipids are constructed exclusively by eukaryotic pathways ***(1)***.

2. Materials

1. TLC plates (20 cm × 20 cm, No. 5721 plate, Merck).
2. TLC chamber.
3. Capillary column (e.g., HR-Thermon 3000B, 0.25 mm × 25 m, Shinwa Chemical Industries, Kyoto).
4. 25–28% ammonia solution, (toxic and corrosive).
5. 0.01% primulin in 80% acetone.
6. I_2.
7. Dittmer-Lester reagent: dissolve 4.01g of MoO_3 in 100 mL of 25N H_2SO_4 by boiling (solution A). Add 180 mg of molybdenum powder of 50 mL of solution A and boil for 15 min. Cool at room temperature and recover the supernatant by decantation (solution B). Mix equal volumes of solution A and B, and dilute threefold with H_2O. Reagent is stable for several months at room temperature ***(2)***.
8. Anthrone reagent: dissolve 0.1 g of anthrone and 2 g of thiourea in 66% H_2SO_4.
9. 5% (w/w) hydrogen chloride methanol solution. This solution is commercially available, but can be prepared by bubbling anhydrous methanol with HCl gas passed beforehand through sulfuric acid for dehydration. When you prepare this

in the laboratory, make a fresh solution every 3–4 mo. This is a toxic, corrosive, and flammable liquid.

10. *n*-Hexane (toxic and flammable liquid).
11. Gas chromatograph.
12. Primulin reagent: 0.01% primulin in 80% acetone.
13. Lipase from *Rhizopus delemar*.
14. A buffer for lipase: 40 m*M* Tris-HCl (pH 7.2), 0.05% Triton X-100.

3. Methods

The methods described here show the isolation of total lipids, the separation into individual lipid classes, and the detection and identification of lipid classes. Additionally, the determination of fatty acid compositions of respective lipid classes and quantification of lipid classes by estimation of fatty acid contents, and the analysis of distribution of fatty acids between *sn*-1 and *sn*-2 positions of glycerol backbones are described.

3.1. Lipid Extraction

Individual lipids can be isolated first as total lipid fraction. Shown below is the method of the isolation according to Bligh and Dyer *(3)* (*see* **Note 1**).

3.1.1. Isolation of Lipids From Algae or Chloroplasts

1. Prepare cells of microalgae or chloroplasts as pellets (*see* **Note 2**) of 0.5–1.0 mL in a 50-mL centrifuge tube with a Teflon-lined screw cap (*see* **Note 3**).
2. Add 10 mL of methanol and agitate it for 30 s with a vortex mixer.
3. Add 5 mL of chloroform and agitate it for 2 min with a vortex mixer.
4. Let stand for 10 min.
5. Add 5 mL of chloroform and agitate it with a vortex mixer for 30 s.
6. Add 5 mL of distilled water and agitate it with a vortex mixer for 30 s. The solution will become turbid.
7. Centrifuge at 1500*g* for 5 min. The solution will separate into three phases; the upper phase of H_2O and methanol, the medium phase of cell debris, and the lower phase of chloroform.
8. Take the lower phase containing lipids with a Pasteur pipet into a flask suitable for the evaporator. Do not contaminate the upper or medium phase.
9. Add 10 mL of chloroform to the remaining upper and medium phases and agitate it for 1 min with a vortex mixer.
10. Centrifuge at 1500g for 5 min. Recover the lower phase of chloroform and combine with the lipid solution prepared in **Subheading 3.1.1., step 8**.
11. Evaporate the solvent completely with rotary evaporator under reduced pressure.
12. Add methanol or chloroform/methanol (2/1, by vol) for complete evaporation if H_2O remains.

13. Dissolve the lipids in an appropriate volume of chloroform/methanol (2/1, by vol), transfer the solution into a tube with a Tefron-lined screw cap, and store it at –20°C until use.

3.1.2. Isolation of Lipids From Leaves of Higher Plants

1. Cut green leaves (5 g of wet weight) into slices.
2. Add 150 mL of chloroform/methanol (1/2, by vol) and homogenize it with a homogenizer.
3. Filtrate the slurry through a piece of filter paper and recover the filtrate in a flask.
4. Add 50 mL of chloroform and agitate it for 1 min.
5. Add 100 mL of 1% KCl solution (w/v) and agitate it for 2 min.
6. Let stand for half a day until the turbid solution separates into three phases.
7. Recover lipids as described in **steps 8–13** in **Subheading 3.1.1.**

3.2. Separation Into Individual Lipid Classes by TLC

TLC can separate individual lipid classes simultaneously on a TLC plate and in a short time (a few hours). For analysis of lipids prepared from plant cells, two-dimensional TLC with two distinct solvent systems is performed, because one-dimensional TLC should fail to separate some lipid classes.

1. Activate a precoated silica gel TLC plate (20 cm × 20 cm, no. 5721 plate, Merck) by heating at 120°C for 1.5 h (*see* **Note 4**).
2. Cool at room temperature.
3. Using a pencil on a TCL plate, outline an oblong spot of approx 5 mm × 15 mm, 2.5 cm away from two neighboring sides of the plate (*see* **Note 5**).
4. Apply lipids dissolved in 20–50 μL of chloroform/methanol (2/1, vol) to the spot with a microsyringe.
5. Develop the plate with the first solvent system, chloroform/methanol/H_2O (65/25/4, by vol) until the front line of the solvent comes 1–2 cm away from the upper side of the plate.
6. Dry the plate by heating the bottom surface with a hair dryer.
7. Develop the plate with the second solvent system, chloroform/methanol/25–28% ammonia solution (13/7/1, by vol) until the front line of the solvent comes 1–2 cm away from the upper side of the plate.
8. Dry the plate by heating the bottom with a hair dryer.

3.3. Detection and Identification of Respective Lipid Classes

Lipid classes separated on a TLC plate must be detected with a non-destructive reagent for further structural analyses, such as a determination of compositions of constituent fatty acids. Primulin and I_2 described in **Subheading 3.3.1.** are amenable to such study, being used for detection of all lipid classes. Zones of the detected lipid classes on the TLC plate are outlined with a pencil

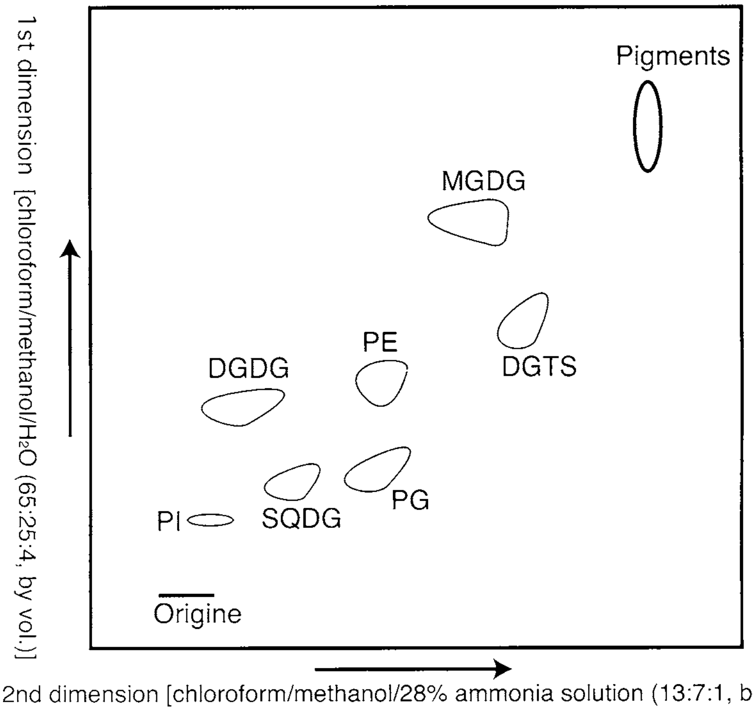

Fig. 1. Separation of total lipids of *C. reinhardtii* cells into individual lipid classes. Abbreviations:DGDG, digalactosyl diacylglycerol; DGTS, diacylglyceryltrimethylhomoserine; MGDG, monogalactosyl diacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyl diacylglycerol.

for their later identification dependent on their mobility and also on staining by reagents that react with particular polar-head groups (*see* **Subheading 3.3.2.**) (*see* **Note 6**). **Figure 1** shows the detected lipid classes prepared from *C. reinhardtii* cells.

3.3.1. Nondestructive Reagents for Detection of All Lipid Classes

3.3.1.1. PRIMULIN REAGENT

1. Spray primulin reagent onto the plate and dry at room temperature.
2. Illuminate it with a long-wave ultraviolet (UV) light (e.g., 365 nm) to detect blue spots of lipid classes.

3.3.1.2. I_2 VAPOR

1. Place a lump of I_2 in a closed box to fill it with I_2 vapor.
2. Stand the TLC plate in the box until brown spots of lipid classes appear.

3.3.2. Destructive Reagents for Detection of Particular Lipid Classes

Outline lipid zones detected in advance by primulin, and treat the zones with Dittmer-Lester or anthrone reagent for detection of phospholipids and glycolipids, respectively, as described below.

3.3.2.1. DITTMER-LESTER REAGENT

1. Spray the reagent onto the plate to detect blue spots of phospholipids.

3.3.2.2. ANTHRONE REAGENT

1. Spray Anthrone reagent onto plate.
2. Heat the plate at 120°C to detect purple spots of glycolipids.

3.4. Determination of Fatty Acid Compositions and Contents of Lipids

It is not necessary to extract respective lipid classes from silica gels of the TLC plates for analysis of their constituent fatty acids. Fatty acid methyl esters are first produced according to the method described in **Subheading 3.4.1.**, then are analyzed by gas-liquid chromatography as in **Subheading 3.4.2.**.

3.4.1. Preparation of Methyl Ester of Fatty Acids

1. Put 50 μL of an internal standard solution such as arachidic acid (1 mg/mL chloroform) into a test tube with a Teflon-lined screw cap and dry it under N_2 stream. This step is necessary for quantification of lipids, but is omitted for determination of fatty acid compositions.
2. Scrape silica gels of lipid zones on TLC with the handle edge of a small spatula, and transfer them to the test tube with a piece of powder paper. Otherwise, transfer lipid fraction such as total lipids dissolved in chloroform/methanol (2/1, by vol) to the test tube, and dry under N_2 stream.
3. Add 2–3 mL of 5% hydrogen chloride methanol solution and close the tube tightly with a screw cap (*see* **Note 7**).
4. Heat at 90°C for 3 h in a heating block with occasional agitation with a vortex mixer, and then cool at room temperature.
5. Add 2–3 mL of *n*-hexane and agitate it for 30 s with a vortex mixer.
6. After letting stand for separation into two phases, transfer the upper *n*-hexane phase containing fatty acid methyl esters into a test tube suitable for evaporation.
7. Evaporate the solvent with a rotary evaporator at 30–40°C.
8. Add 2–3 mL of *n*-hexane to the remaining lower phase in **Subheading 3.4.1., step 6**, and agitate it for 30 s with a vortex mixer.
9. After letting it stand for the separation, transfer the upper phase to the test tube in **Subheading 3.4.1., step 7**, and evaporate the solvent.
10. Repeat **steps 8–9** above.
11. Dissolve the methyl esters in 50 μL of *n*-hexane.

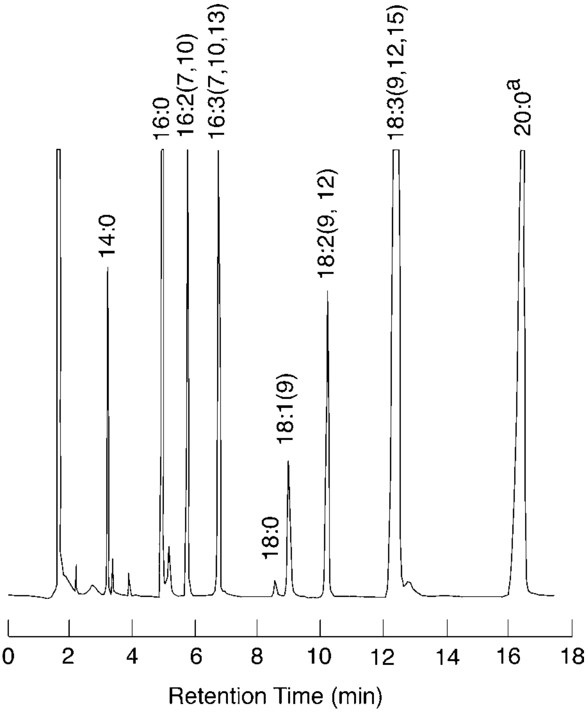

Fig. 2. Separation of fatty acid methyl esters prepared from monogalactosyl diacylglycerol of *C. reinhardtii*. Fatty acyl groups are denoted by the numbers of carbon atoms and double bonds, e.g., 18:1(9) = oleoyl. [a]An internal standard.

3.4.2. Analysis of Fatty Acid Methyl Esters With Gas-Liquid Chromatography

1. Set up a gas chromatograph equipped with a capillary column (*see* **Note 8**). Temperatures of injector and detector of the flame ionization are 250°C, while that of the column is 180°C.
2. Inject 1 µL of the methyl ester solution.
3. Record and store chromatograms with a chromatography data processor. **Figure 2** shows a separation pattern of fatty acid methyl esters prepared from DGDG of *Chlorella kesslerii* (*see* **Note 9**). Peak areas that correspond to weights of fatty acid methyl esters are calibrated by respective molecular weights for determination of molar ratios and compositions of fatty acids.
4. Total fatty acid contents of each lipid are calculated through multiplication of a molar ratio of total constituent fatty acids to an internal standard by the known content of the internal standard. Lipid contents can thus be estimated by division of the total fatty acid contents by two.

3.5. Determination of Fatty Acid Compositions at sn-1 and sn-2 Positions of Individual Lipid Classes

Lipases that liberate fatty acids from lipids are classified into phospholipid-specific lipases and nonspecific lipases. Described below is the method of nonspecific lipase from *Rhizopus delemar* that hydrolyzes ester bond at the *sn*-1 position of diacylglycerol moiety ***(4)***.

1. Scrape off silica gels containing a lipid class separated by TLC into a test tube.
2. Add 2–3 mL of chloroform/methanol (2/1, by vol) and agitate the tube for 2 min for the lipid extraction.
3. Centrifuge at 1500*g* for 5 min and recover the supernatant into a test tube suitable for evaporation.
4. Evaporate the solvent completely with a rotary evaporator
5. Add 0.9 mL of 50 m*M* Tris-HCl, pH 7.2 and 0.05% Triton X-100 and agitate the tube with a vortex mixer. Sonicate the solution for 5 min until the lipids are completely dissolved.
6. Add 0.5 mg of lipase from *Rhizopus delemar* (Seikagaku corporation) dissolved in 0.1 mL of 50 m*M* Tris-HCl, pH 7.2.
7. Incubate at 37°C for appropriate times (e.g., 10 min for monogalactosyl diacylglycerol, 20 min for digalactosyl diacylglycerol, 30–60 min for sulfoquinovosyl diacylglycerol or phosphatidylglycerol, 90 min for phosphatidylethanolamine, and 360 min for phosphatidylcholine) (*see* **Note 10**).
8. Add 1 mL of chloroform/methanol (1/1 by vol) and agitate it for 1 min to extract products by the lipase.
9. Recover the lower phase and evaporate the solvent.
10. Dissolve the lipids in 50 mL of chloroform/methanol (2/1 by vol), and develop it by one-dimensional TLC with the solvent system of chloroform/methanol/H_2O (65/25/4, by vol).
11. Spray primulin reagent to detect *sn*-2-acyl-lysolipids, original lipids, and released fatty acids. The *sn*-2-acyl-lysolipids migrates slower than original lipids, while fatty acids move faster, positioned close to the front line of the solvent.
12. Analyze fatty acid methyl esters of the *sn*-2-acyl-lysolipids by gas-liquid chromatography for determination of fatty acid compositions at the *sn*-2 positions, according to the method described in **Subheading 3.4.** Fatty acid compositions at the *sn*-1 positions can be estimated by comparison of fatty acid compositions between original lipids and *sn*-2-acyl-lysolipids.

4. Notes

1. Use glass-made instruments for isolation and storage of lipids, because chloroform and methanol elute plasticizer of plastic instruments.
2. Samples for isolation of total lipids can be stored not at –20°C, but at –80°C, for repression of breakdown of lipid molecules by endogenous lipases.
3. A larger pellet requires increases in volumes of reagents for subsequent lipid isolation.

4. Activated TLC plates can be stocked in a desiccator, but activation just before performance of TLC is recommended especially in places of high humidity.
5. The spot of lipids as an origin on the TLC plate should not be so big as to repress increases in the sizes of respective lipid zones. Silica gel will perturb subsequent fatty acid analysis by gas-liquid chromatography, since the TLC plate is contaminated with saturated fatty acids such as 16:0 and 18:0.
6. The reagents for detection of lipids with particular polar-head groups destroy lipid molecules, therefore are not useful for their subsequent structural analyses.
7. Be careful not to use a cracked tube in case that the tube explodes.
8. Chromatograph equipped with a capillary column is recommended owing to the high performance in the separation of almost all fatty acid methyl esters, including isomers such as 18:1(9) and 18:1(11).
9. Molecular species of fatty acid methyl esters can be identified through comparison of retention times on chromatograms with fatty acid methyl ester standards that can be prepared by methanolysis of commercially available fatty acids.
10. Fatty acids at the *sn*-2 positions of the lysolipids will be transferred to the *sn*-1 positions and be hydrolyzed by the action of the lipase. Thus, fatty acids to be analyzed should be *sn*-2-acyl-lysolipids, but not released fatty acids, and reaction should be stopped before complete breakdown of original lipids.

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17

DNA Adducts With Chlorophyll and Chlorophyllin As Antimutagenic Agents

Synthesis, Stability, and Structural Features

Heidar-Ali Tajmir-Riahi, Jean-Francois Neault,
and Stavroula Diamantoglou

Summary

Porphyrins and their metal derivatives are strong DNA binders with association constants of $10^5\,M^{-1}$ to $10^7\,M^{-1}$. Some of these compounds have been used for radiation sensitization therapy of cancer and are targeted to interact with cellular DNA. Chlorophyll (CHL) and chlorophyllin (CHLN), a food-grade derivative of chlorophyll, the ubiquitous green plant pigment widely consumed by humans, are potent inhibitors of experimental carcinogenesis. The aim of this report was to examine the interaction of calf-thymus DNA with CHL and CHLN in aqueous solution at physiological pH, with pigment/DNA(phosphate) molar ratios (r) of 1/80 to 1/2. Fourier transform infrared (FTIR) difference spectroscopic method was used to determine the pigment binding mode, binding constant, sequence selectivity, DNA secondary structure and structural variations of the pigment–DNA complexes in aqueous solution.

Spectroscopic evidence showed that chlorophyll is an external DNA binder with no affinity toward DNA intercalation. CHL bindings are through the backbone PO_2 group and the guanine N-7 site of the G-C base pair (major groove) with overall binding constant of $K = 1.13 \times 10^4$ M^{-1} CHLN binds DNA via intercalative mode into the G-C and A-T-rich regions with a minor perturbation of the backbone PO_2 group with overall binding constant $K = 3.56 \times 10^3\ M^{-1}$. However, The CHL distributions are 60% with the backbone PO_2 group and 20% with the G-C base pairs, whereas CHLN intercalation is 25% with A-T and 15% with G-C base pairs. A partial reduction of B-DNA structure in favor of A-DNA occurs upon CHL and CHLN complexation.

Key Words: DNA; chlorophyllin; chlorophyll; antimutagen; drug; binding mode; binding constant; conformation; FTIR spectroscopy helix stability.

1. Introduction

Diet plays an important role in human cancer risk ***(1–4)***. Although attention has focused on dietary carcinogens and promoters, recent evidence shows that

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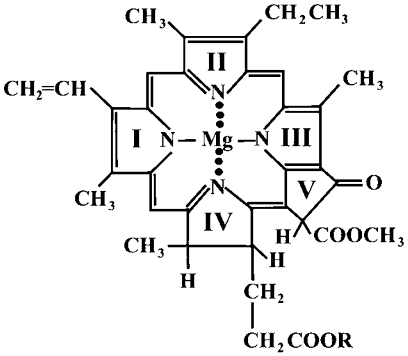
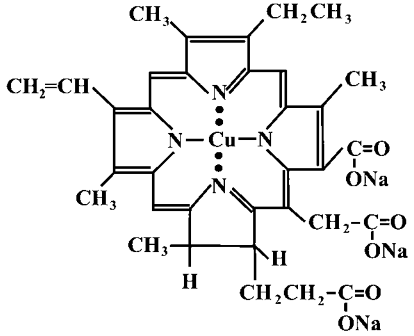


CHLOROPHYL a



CHLOROPHYLLIN

Structure 1

protective factors within the diet are also important in cancer risk. Several chemical components of fruits and vegetables have been isolated and purified and shown to be effective against carcinogenesis in experimental animal models *(5–8)*. Chlorophyll (CHL) (**Structure 1**) and its derivatives have been shown to exert profound antimutagenic behavior against a wide range of potential human carcinogens *(7–9)*. Similarly, chlorophyllin (CHLN) (**Structure 1**), a food-grade derivative of chlorophyll, has been used historically in the treatment of several human conditions, with no evidence of human toxicity *(10–12)* and recently it was used as a potent inhibitor of aflatoxin B_1 hepatocarcinogenesis in rainbow trout *(13)*.

It has been suggested that the antimutagenic activity of chlorophyllin comes from its strong complexation with parent mutagens or their intermediates, scavenging of free radicals and active oxygen species, and suppression or interference with metabolic activation by specific cytochrome (P450) and other metabolizing enzymes ***(14–17)***. CHLN was also shown in vivo to efficiently inhibit precarcinogenic target organ DNA adduction by aflatoxin B_1 in trout ***(16)*** and 2-amino-3-methylimidazol [4,5-f] quinoline in rat ***(18)***. Because the major target of these anticancer drugs can involve DNA or DNA adducts, the interaction of DNA with CHL and CHLN has major biological importance and thus, the present study is designed to investigate pigment–DNA complexation in vitro and to provide structural information regarding the pigment binding mode, sequence preference, and DNA secondary structure, using infrared spectroscopy. Our structural information provides, to our knowledge, the first spectroscopic evidence regarding CHL–DNA and CHLN–DNA interactions and may elucidate the nature of this biologically important complex formation.

More recently, vibrational spectroscopic method has been used to analyze the nature of DNA–drug complexation and to provide structural information at molecular level ***(19–20)***. Recently, we used vibrational spectroscopy (infrared and Raman) for the structural characterization of diethylstilbestrol (DES)–DNA, aspirin–DNA, vitamin–DNA and carbohydrate–DNA ***(21–24)*** as well as cation–DNA ***(25,26)*** and protein complexes ***(27)***. We believe that FTIR difference spectroscopic method can be also applied here, in order to characterize the nature of the pigment–DNA interaction.

The goal of this chapter is to describe the synthesis, separation, and FTIR difference spectroscopic method for DNA complexation with CHL and CHLN in aqueous solution at physiological pH, with pigment/DNA(P) molar ratios of 1/80 to 1/2. Spectroscopic evidence regarding the pigment-binding mode, binding constant, sequence selectivity, and biopolymer secondary structure is provided. Furthermore, comparisons were made between the CHL–DNA and CHLN–DNA complexes and the corresponding metalloporphyrin–DNA adducts and the results are reported here. This study elucidates the nature of this biological interaction and the possible antimutagenic role of CHL and CHLN.

2. Materials

1. 200 mg highly polymerized type I calf-thymus DNA sodium salt (7% Na content) (Sigma).
2. 2 mg crystalline chlorophyllin (copper-trisodium salt) (Sigma).
3. 2 mg crystalline chlorophyll *a* (Sigma).
4. 1 g crystalline sodium cacodylate (Sigma).
5. Ethanol (100%).
6. Preparation of Pigment–DNA Complexes.

Sodium-DNA was dissolved to 2% w/w (0.05 *M* DNA [phosphate]) in 0.05 *M* NaCl and 1 m*M* sodium cacodylate (pH 7.30) at 5°C for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The appropriate amount of chlorophyll or chlorophyllin (0.3–12.5 m*M*) was prepared in distilled water and added dropwise to DNA solution to attain desired pigment/DNA(P) molar ratios of 1/80, 1/40, 1/20, 1/10, 1/4, and 1/2 at a final DNA concentration of 1% w/w or 0.025 *M* DNA (phosphate) (*see* **Note 1**). The solution pH was adjusted to 7.30–6.80, using NaOH solution. The infrared spectra were recorded 3 h after mixing of pigment and DNA solutions. The infrared spectra of pigment–DNA complexes with $r > 1/2$ could not be recorded in solution due to solid gel formation (*see* **Note 2**).

3. Methods

3.1. FTIR Measurements

The infrared spectra were recorded on a Bomem DA3-0.02 FTIR spectrometry equipped with a nitrogen-cooled HgCdTe detector and KBr beam splitter. The solution spectra were taken using AgBr windows with resolution of 4 cm^{-1} and 100–500 scans. The sample preparation and spectral measurements were performed under green light (to avoid photodegradation of chlorophyllin or chlorophyll in pigment–DNA complexes). The water subtraction was performed as in our previous report *(21,22)*. A good water subtraction is achieved as shown by a flat baseline around 2200 cm^{-1} where the water combination mode *(28)* is located (*see* **Note 3**). The difference spectra [(DNA solution + pigment solution)–(DNA solution)] were produced, using a sharp DNA band at 968 cm^{-1} as internal reference. This band, owing to deoxyribose C-C stretching vibrations, exhibits no spectral changes (shifting or intensity variations) on pigment–DNA complexation and was cancelled upon spectral subtraction. The intensity ratios of several DNA in-plane vibrations, related to A-T and G-C base pairs and the PO_2 stretching modes were measured with respect to the reference band at 968 cm^{-1}, as a function of pigment concentration with error of ± 2%. These intensity ratio measurements are used to quantify the amounts of pigment bindings to the backbone PO_2 group and DNA bases. The detailed infrared spectral manipulations and intensity ratio calculations are presented in our recent publications *(21–26)*.

3.2. Analysis of CHL–DNA Complexes

The binding of CHL with DNA duplex is shown at different pigment concentrations (1/80 to 1/2) in **Fig. 1A** and **Fig. 2A**. CHL binding is to the backbone PO_2 group and indirectly (via H_2O) to the N-7 site of the guanine bases through the central Mg cation. Evidence for direct cation-phosphate coordination is concluded from the major intensity increase (50%) of the PO_2 antisymmetic stretching band at 1222 cm^{-1} together with the shift of this vibration toward a lower frequency at 1220 cm^{-1} (**Fig. 1A** and **Fig. 2A**). Similarly, the guanine band at 1717 cm^{-1} *(28–31)* gains in intensity (25%) and shifts toward a lower frequency at 1715 cm^{-1} that results from an indirect Mg–N-7 interaction via H_2O (**Fig. 1A** and **Fig. 2A**). The bands at 1663 cm^{-1} (mainly thymine) showed increase in intensity (30%) with no spectral shifting upon CHL interaction, whereas the bands at 1609 (adenine) and 1485 cm^{-1} (mainly cytosine) exhibited a minor increase in intensity with no frequency shift in the spectra of the CHL–DNA complexes (**Fig. 1A** and **Fig. 2A**). The observed spectral changes show a direct Mg–PO_2 binding with an indirect Mg–H_2O N-7 coordination to the guanine bases. However, a minor indirect cation interaction via H_2O to thymine O-2 atom can also be included, whereas, metal ion binding to the adenine and cytosine bases is negligible. It is worth mentioning that at this stage of complexation, the Mg–DNA binding sites are not involved in hydrogen bonding and therefore this type of complexation does not bring about helix destabilization. It should be noted that the direct Mg–PO_2 coordination and indirect Mg–N-7 (guanine) interaction through H_2O are found in the crystal structure of d(CpGpCpGpCpG) oligonucleotide, stabilizing left-handed Z-DNA conformation ***(32)***. On the basis of the spectroscopic results, the Mg cation binding to the backbone PO_2 and the guanine N-7 sites are also suggested for the Mg–ATP complexes with calf-thymus DNA in hydrated films ***(33)***. Raman spectroscopic studies also showed direct Mg-PO_2 binding for the Mg–DNA complexes in aqueous solution ***(34)***. On the basis of the FTIR spectroscopy, direct and indirect Mg cation bindings to the phosphate group and the base N-7 atom of guanine or adenine were found in the solid state and solution structure of the Mg–guanosine-5'-monophosphate (GMP), Mg–deoxyguanosine-5'-monophosphate dGMP) and Mg–adenosine-5'-monophosphate (AMP) complexes ***(26,35,36)***.

3.3. CHLN–DNA Complexes

Figures 1B and **2B** show how CHLN binds DNA at different pigment concentrations (r = 1/80 to 1/2). CHLN binds via intercalative mode into the G-C and A-T regions. Evidence for this comes from the minor increase in the intensity (5%) of the mainly G band at 1717 cm^{-1} and T band at 1663 cm^{-1} ***(28–31)*** (**Fig. 1B** and **Fig. 2B**). These intensity changes were associated also with the shift of the bands at 1717 (mainly G) to 1715 and 1663 (mainly T) to 1666 cm^{-1}. Similarly, the mainly adenine band at 1609 shifted toward a lower frequency at 1605 cm^{-1}. A minor intensity increase (5%) was also observed for the backbone PO_2 antisymmetric stretching vibration at 1222 cm^{-1} upon CHLN complex-ation (**Fig. 2B**). The spectral changes observed for the phosphate band at 1222 cm^{-1} are also owing to some degree of Cu-PO_2 interaction (outside binding). The positive derivative features observed at 1710, 1660, and 1214 cm^{-1}, in the difference spectra of CHLN–DNA complexes, are resulting from the increase in intensity of the bands at 1717, 1663, and 1222 cm^{-1} (**Fig. 1B** and **Fig. 2B**).

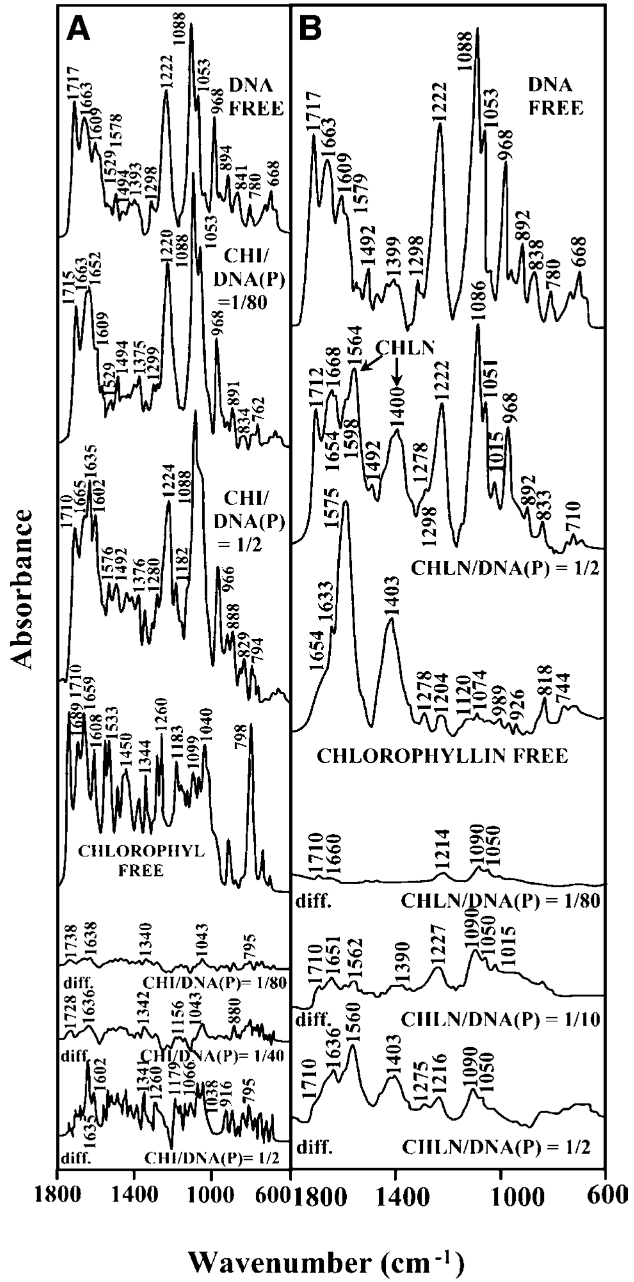


Fig. 1. FTIR spectra and difference spectra [(DNA solution + pigment solution)–(DNA solution)] of the free calf-thymus DNA and its pigment adducts for chlorophyll (**A**) and chlorophyllin (**B**) in aqueous solution at pH = 6.8–7.30 with different pigment/DNA(P) molar ratios in the region 1800–600 cm^{-1}.

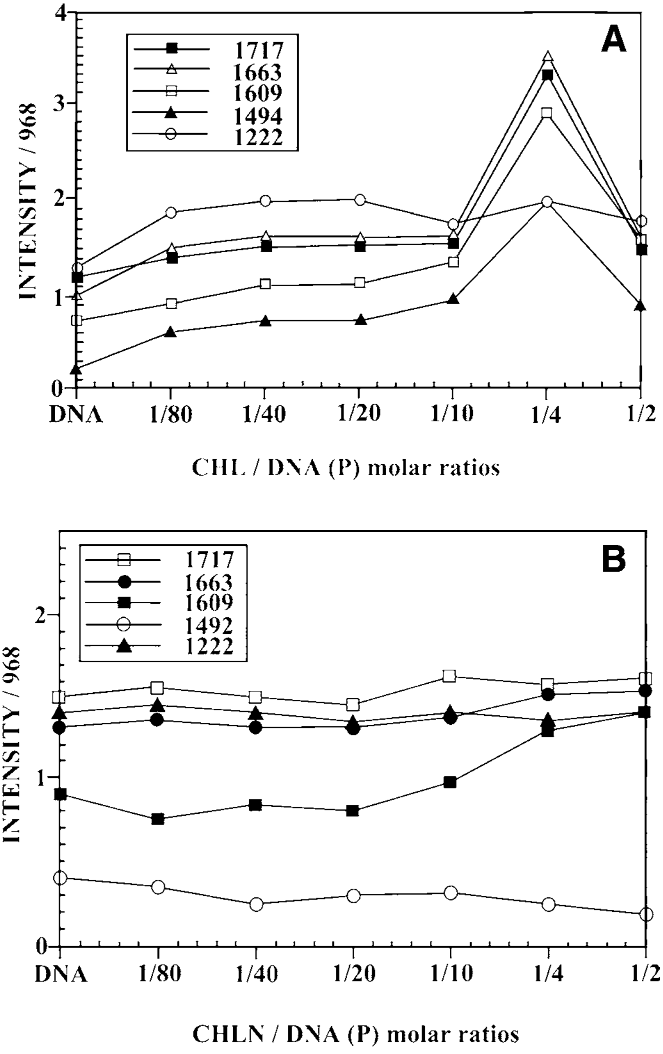


Fig. 2. Intensity ratio variations for several DNA in-plane vibrations at 1717 (G,T), 1663 (T,G,A,C), 1609 (A), 1492 (C,G) and 1222 cm^{-1} (PO_2 stretch) as a function of pigment concentrations for chlorophyll (**A**) and chlorophyllin (**B**).

3.4. Calculation of Binding Constants

Calculation of the binding constants was carried out as reported for other pigment complexes with DNA, RNA, and mononucleotides *(37–40)*. By assuming that there is only one type of interaction (intercalation) between pigment and DNA molecule, the following equations can be established.

$$\text{pigment} + \text{DNA} \Leftrightarrow \text{pigment} : \text{DNA} \qquad \text{Eq. 1}$$

$$K = [\text{pigment: DNA}] / [\text{pigment}]\,[\text{DNA}] \qquad \text{Eq. 2}$$

$$\text{pigment} = \text{CHL or CHLN}$$

Because CHLN intercalation is limited to the G-C and A-T bases, the relative intensity variations of the bands at 1717 cm^{-1} (mainly G), at 1663 cm^{-1} (mainly T) were calculated for each drug concentration. The calculated intensities were used as a function of pigment concentration to estimate the K(G) for guanine and K(T) for thymine bases. The double reciprocal plot of $1/(I-I_0)$ vs 1/[pigment] is linear and the binding constant (K) can be estimated from the ratio of the intercept to the slope. I_0 is the initial relative intensity of the specific DNA absorption band and I is the relative intensity at different pigment concentrations. The overall binding constants (K) are estimated to be $3.56 \times 10^3\ M^{-1}$ for CHLN-DNA and $1.13 \times 10^4\ M^{-1}$ for CHL–DNA complexes. The estimated k values show stronger CHL-DNA interaction than the CHLN–DNA complexes. Similar methods, based on the intensity ratio variations of the Raman and infrared vibrational frequencies, have been used to determine the binding constants of diethylstilbestrol (intercalating drug) ***(19)*** to polynucleotides and methylmercury cation to mononucleotides ***(37)***.

The value of $K = 3.56 \times 10^3\ M^{-1}$ estimated for the DNA–CHLN complexes is consistent with the chlorophyllin being a weak DNA-intercalator, whereas CHL is a major groove binder (*see* **Note 4**). The association constants of the CHLN–DNA complexes are smaller than those of the strong DNA intercalators, such as ethidium bromide, acridine orange, methylene blue, and other pigments with binding constants ranging $10^5\ M^{-1}$ to $10^6\ M^{-1}$ ***(38–39)***. Similarly, the K values for CHL-DNA adducts are smaller than those of the simple cation-DNA complexes ***(40)***.

3.5. Pigment Distributions

Based on the $K = 1.13 \times 10^4\ M^{-1}$ for the CHL–DNA complexes, CHL was bound 60% to G-C and 25% to the backbone phosphate group (**Fig. 3A**). However, chlorophyllin distributions were 20% with A-T and 15% with G-C base pairs and less than 10% around the backbone phosphate group (based on the binding constant $K = 3.5 \times 10^3\ M^{-1}$) (**Fig. 3B**).

3.6. DNA Conformation

Spectral changes were associated with the loss of the intensity and shifting of the B-DNA indicator at 838 to 833 (CHLN) and 829 cm^{-1} (CHL), in the spectra of the pigment–DNA complexes (**Fig. 1A,B**). The shift of the B-DNA indicator ***(25,30)*** from 838 to 833-829 cm^{-1} was accompanied by the shift of the other B-DNA marker band at 1717 to 1712 and 1710 cm^{-1} (**Fig. 1A,B**). The

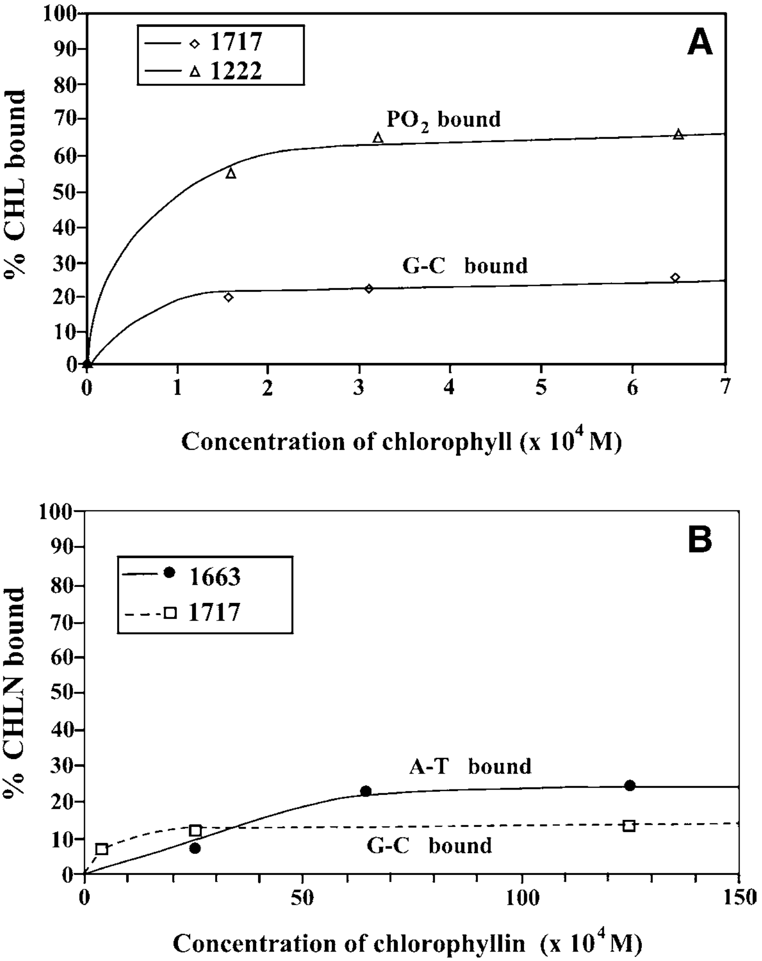


Fig. 3. Calculated distributions of the pigment bound to the A-T (1663 cm^{-1} mainly thymine) and to the G-C (1717 cm^{-1} mainly guanine) and backbone phosphate (1222 cm^{-1}) in aqueous solution with DNA concentration of 0.025 *M* (phosphate) as a function of pigment concentration (*M*) for chlorophyll **(A)** and chlorophyllin **(B)** based on the $K = 1.13 \times 10^4\ M^{-1}$ (CHL) and $K = 3.56 \times 10^3\ M^{-1}$ (CHLN) at pigment concentration of $6.5 \times 10^{-3}\ M^{-1}$.

observed spectral changes are related to a partial reduction of B-DNA structure towards A-DNA, upon CHL and CHLN interactions

CHLN, with four-coordinate copper cation in the center is a good candidate for DNA intercalation, although Chl with five- or six-coordinate magnesium ion (in aqueous solution) binds DNA via nonintercalative mode. Based on the infrared spectroscopic results presented here for the first time, it is evident that CHLN binds DNA via intercalative mode into the G-C and A-T-rich regions with minor pigment–PO_2 interaction (outside binding). At high pigment content, minor helix destabilization occurs with a partial reduction of the B-DNA structure in favor of A-DNA. On the other hand, CHL binding with DNA was mainly through Mg–PO_2 interaction (outside binding) with minor Mg–N-7 coordination (major groove binding). CHL complexation resulted also in a major reduction of B-DNA structure in favor of A-DNA with a partial helix opening at high pigment content. The calculated binding constants are $K = 1.13 \times 10^4$ M^{-1} for CHL–DNA and 3.56×10^3 M^{-1} for CHLN–DNA adducts. The differences in the stability of these pigment–DNA complexes can be attributed to a stronger interaction of Mg–PO_2 (external binding) in the CHL–DNA and a weak intercalative mode of the chlorophyllin in the CHLN–DNA complexes (*see* **Note 5**). The larger size of the CHLN (with respect to CHL) plays an important role in the formation of less stable metalloporphyrin–DNA complexes.

4. Notes

1. CHLN is soluble in water but not CHL. Chlorophyll was dissolved in ethanol and then added to DNA solution.
2. At higher pigment concentrations, pigment aggregation does not favor DNA complexation and lead to gel formation.
3. Water subtraction was carried out based on **ref. *28***. This method is a rough estimate, but removes the water effectively.
4. The stronger CHL–DNA interaction allowed us to calculate the specific binding constants to guanine N-7 with $K(G) = 4.4 \times 10^3$ M^{-1} and to the backbone PO_2 group with $K(P) = 2.90 \times 10^4$ M^{-1}. This shows that the primary target of CHL is the backbone phosphate group (strong binding) and secondary binding at guanine bases (weak binding).
5. Spectral changes observed for intercalating drug such as CHLN are less pronounced than those of the major groove DNA binding such as CHL (*see* **Fig. 2A,B**).

Acknowledgments

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18

Incorporation and Analysis of LHCII in Model Systems

Wieslaw I. Gruszecki

Summary

The largest light-harvesting pigment-protein complex of Photosystem II (LHCII) is an antenna complex comprising more than half of photosynthetically active chlorophyll pigments. Understanding photophysical processes in this pigment-protein complex and the relationship between the structure and function of LHCII has been the subject of numerous studies, including those in the model systems, such as monomolecular layers, Langmuir-Blodgett films, planar lipid bilayers, and liposomes. This chapter describes the methods of the incorporation of LHCII into these particular model systems, control of intactness of LHCII after incorporation, and the examination of this pigment-protein complex in model systems.

Key Words: Langmuir-Blodgett films; LHCII; liposomes; monolayers; planar lipid membranes.

1. Introduction

The largest light-harvesting pigment–protein complex of photosystem II (LHCII) has been the subject of numerous studies. Various studies have been carried out in vivo or isolated from the photosynthetic membranes but also incorporated to various model systems such as monomolecular layers, Langmuir-Blodgett (L-B) films, liposomes, and planar lipid bilayers. All the model systems listed above are very different, in terms of molecular structure, and a selection of particular model system depends on the nature of scientific problem one is going to investigate.

1.1. Monolayers

A monomolecular layer (monolayer) technique has been frequently applied in studying molecular properties of lipids *(1)*. In most cases, a monolayer is formed at the air–water interface by deposition of a solution of amphiphilic molecules at the interface. A solid monolayer is formed by compressing the layer, after the time required for the evaporation of molecules of a deposition solvent.

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The situation is different in the case of monomolecular layers formed with proteins. Most organic solvents suitable for deposition of lipid monolayers are not suitable for preparing a protein solution, owing to the risk of denaturation. On the other hand, solvents appropriate for preparing protein solutions are usually miscible with the aqueous subphase and therefore a monolayer may not be easily deposited at the air–water interface. In contrast to lipid monolayers, a formation of a protein monolayer requires an individual procedure for each protein. The main requirement for a deposition procedure is to preserve native protein structure associated with physiological functions examined with the application of the monomolecular layers technique. A deposition solvent has to dissolve bulk protein aggregates, on the one hand, and also allow deposition of protein molecules at the air–water interface. The method elaborated for LHCII is described in detail below.

1.2. Langmuir-Blodgett Films

Monomolecular layers at the air–water interface are often not suitable for spectroscopic measurements or measurements carried out with the application of microscopic techniques. Monolayers compressed at the interface to a certain surface pressure, can be deposited to solid support, such as glass or mica, by means of L-B technique *(2)*. The selection of a solid support for deposited monolayers (L-B films) is associated with the nature of experiments conducted with these samples. For example, films deposited to glass and in particular to quartz slides are suitable for electronic absorption and fluorescence spectroscopy *(3,4)* those deposited to mica are suitable for scanning force microscopy *(5,6)* and those deposited to silicon or to a monocrystal of ZnSe are suitable for infrared absorption spectroscopy *(3,7)*. These requirements apply for L-B films formed with LHCII as will be presented below.

1.3. Liposomes

In several cases, LHCII incorporated to bimolecular lipid membranes appears to be a good model of studying functions of this pigment–protein complex. Both small unilamellar liposomes or large multilamellar liposomes may be applied as a convenient and easy-to-prepare model of lipid bilayers, depending on the nature of a scientific problem addressed. For example, the effects of lipids on spectroscopic properties of LHCII-bound photosynthetic pigments may be analyzed with the application of multilamellar liposomes *(8)* but the effect of LHCII on ion transport across lipid membranes has to be analyzed in the model system composed of unilamellar vesicles *(9)*. In such a case, ion-sensitive dyes or fluorescence labels trapped inside liposomes can be applied to monitor ion transport across the membrane (from the outer to the inner compartment of liposomes). The procedures of incorporation of LHCII to liposomes is described below.

1.4. Planar Lipid Biolayers

The problem of the effect of LHCII on lipid membrane permeability for ions can be also addressed with the application of planar lipid bilayer model, also called black or bimolecular lipid membranes (BLM) ***(9)***. Planar lipid bilayer is a model suitable for different kinds of electrical measurements, in particular when the membrane separates two aqueous phases where electrodes may be placed. The effective procedure of incorporation of LHCII into BLMs involves two steps: the incorporation of a protein to liposomes, followed by the fusion of LHCII-containing liposomes with the planar lipid membrane ***(9,10)***. The details of this method are presented below.

2. Materials

2.1. Monolayers of LHCII Deposited at the Air–Water Interface

1. Water used as a subphase and for preparation of the buffer was redistilled and distilled a third time from $KMnO_4$ in order to remove possible organic contaminations. The deposition medium was composed of 2-propanol and Tricine buffer (10 m*M*, pH 7.6 containing 10 mM KCl) in the proportion of 1:4 (by volume). 2-Propanol for preparation of a deposition solution was of analytical grade. The air phase above the subphase has to be enriched with or exchanged for a neutral gas such as nitrogen or argon in order to remove molecular oxygen and avoid oxidative degradation of LHCII during monolayer deposition and compression.
2. Langmuir-Blodgett films: Supports for deposition of L-B films may be effectively cleaned with the so-called “piranha solution” composed of H_2O_2 (30%) and H_2SO_4 (100%) in the proportion of 1:2 (by volume). Exceptional caution has to be paid while handling this hazardous mixture. After removing slides from this solution and extensive washing with redistilled water, the samples are kept in redistilled water alkalized with KOH solution to neutral pH values. Before use, solid supports are additionally kept for at least 30 min under reduced pressure (less than 10^{-5} bar) in order to remove remaining impurities and to prevent possible contamination.
3. Liposomes: Liposomes can be formed out of commercial lipids such as egg yolk phosphatidylcholine or lipids isolated from plant material such as digalactosylodiacyloglycerol. Liposomes are formed in the 10 m*M* Tricine or Bis-Tris buffer, pH range 7.2–7.6, containing 10 m*M* KCl.
4. Planar lipid bilayers: Planar bilayer lipid membranes can be formed from commercial lipids such as egg yolk phosphatidylcholine or asolectin dissolved in *n*-decane (7 mg/mL). Bathing solutions are composed of 10 m*M* Bis-Tris buffer, pH 7.2 and KCl in the range 0.02 to 0.22 *M*. LHCII-containing liposomes were formed in a buffer solution as described above except that 0.4 *M* glycerol was additionally present in order to create an osmotic tension across a liposome membrane, required for membrane fusion.

3. Methods

3.1. LHCII in Monolayers

1. Form LHCII monolayers ***(5,6,11)*** in a Teflon trough (e.g., 10 × 40 cm, ***(5,6)*** and compress with a Teflon barrier. The optimal rate of compression is between 5 cm^2 min^{-1} and 20 cm^2 min^{-1}.
2. Monitor surface pressure with the application of Langmuir method or Wilhelmy plate method. In both cases commercially available tensiometers connected to a computer acquisition system can be applied.
3. Spread LHCII at the argon–water interface using a nondestructive concentration of 25% 2-propanol. This solvent was selected among several water miscible organic solvents as one that did not lead to pigment extraction and protein denaturation at concentrations required to disintegrate bulk aggregates of LHCII.
4. Test integrity of LHCII by analysis of the efficiency of the excitation energy transfer between chlorophyll *b* and chlorophyll *a*. **Figure 1** depicts chlorophyll fluorescence emission spectra of LHCII in a buffer containing different concentration of 2-propanol, excited in the absorption maximum of chlorophyll *a* (440 nm) and chlorophyll *b* (470 nm). The existence of a single emission band characteristic of chlorophyll *a*, centered at 680 nm is indicative of efficient excitation energy transfer from chlorophyll *b* to chlorophyll *a*. As can be seen, relatively high concentrations of alcohol disintegrate the complex to a level that affects excitation energy transfer. This is demonstrated by the appearance of an additional emission band in the region of 660 nm (while excited at 470 nm), characteristic of chlorophyll *b*. **Figure 2** presents the results of the same kind of fluorescence measurements carried out with single monolayer of LHCII formed at the argon–water interface and deposited afterwards to quartz support by means of L-B technique.
5. Spread the protein suspension in a buffer supplemented with 25% (by volume) 2-propanol at the argon–water interface. **Figure 3** depicts the surface pressure-molecular area isotherm of compression of LHCII monolayer.
6. Fit the linear part of the isotherm with a line and extrapolation of this line to zero surface pressure gives the specific molecular area of LHCII close to 1450 $Å^2$. Such a value corresponds well with the molecular dimensions of LHCII: an elliptical cross-section 30 Å × 50 Å ***(12)***. From this cross-section a molecular area in the range of 1180 $Å^2$ (the elliptical surface) and 1500 $Å^2$ (the rectangular surface) may be expected, taking into consideration molecular packing phenomena at the interface.
7. Deposit L-B films of LHCII onto a solid support preferably within the surface pressure range from 15 mNm^{-1} to 20 mNm^{-1} with the speed of 5 cm^2 min^{-1} or less (total surface of the slide or other support per time unit) using a commercial or homemade film-lift ***(3–6)*** (*see* **Note 2**).
8. Compress the monolayer during the deposition, preferably with the same speed as the speed of the movement of the solid support. **Figure 4** presents atomic force microscopy image of the topography of LHCII monolayer deposited to mica by means of L-B technique.

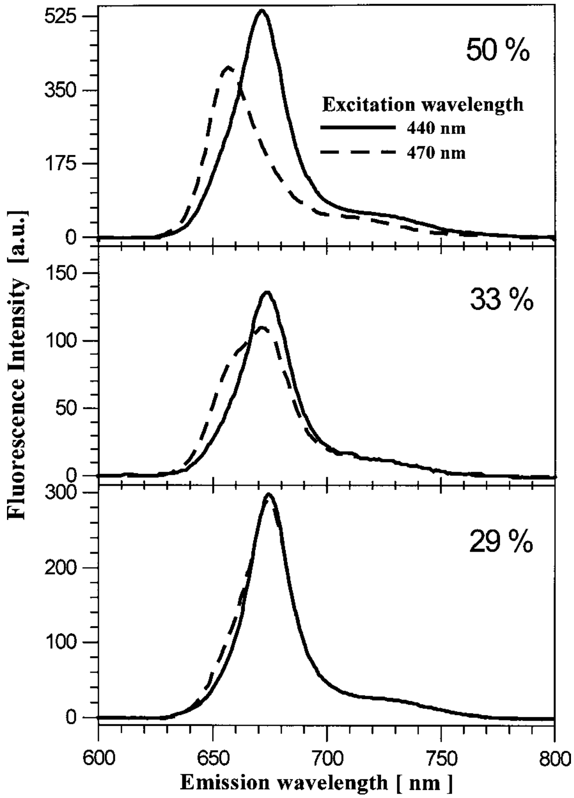

Fig. 1. Fluorescence emission spectra recorded from LHCII suspension in a Tricine buffer (10 m*M*, pH 7.6, containing 10 m*M* KCl) supplemented with 2-propanol in a concentration 50, 33, and 29% (by volume, indicated). Excitation wavelengths at 440 nm (solid line) and at 470 nm (dashed line) correspond to chlorophyll *a* and chlorophyll *b*, respectively.

3.2. LHCII in Liposomes

1. Liposomes containing incorporated LHCII were prepared at lipid concentration in a buffer ranging from 1 mg/mL *(9)* to 10 mg/mL *(8)*.
2. The thin film of a lipid is formed at the bottom of glass tube by evaporation from chloroform or ethanol.
3. The lipid film is kept under reduced pressure (less than $1/10^5$ bar) for at least 30 min in order to remove the possible remaining traces of an organic solvent.
4. The tube is filled with the required volume of the buffer and small liposomes are formed by sonication with ultrasonic disintegrator (5 min at frequency 22 kHz, amplitude 1–8 μm) or extrusion procedure with the application of polycarbonate Millipore membranes (*see* **Note 3**).

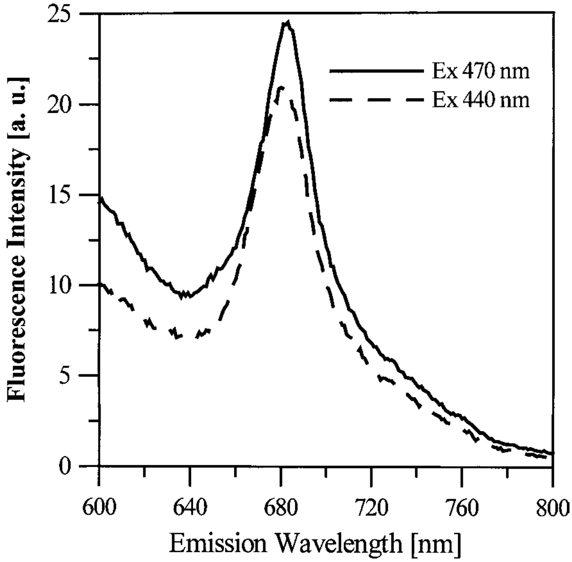

Fig. 2. Fluorescence emission spectra recorded from a single LHCII monolayer deposited to quartz slide by means of Langmuir-Blodgett technique. Excitation wavelengths at 440 nm (dashed line) and at 470 nm (solid line) correspond to chlorophyll *a* and chlorophyll *b*, respectively.

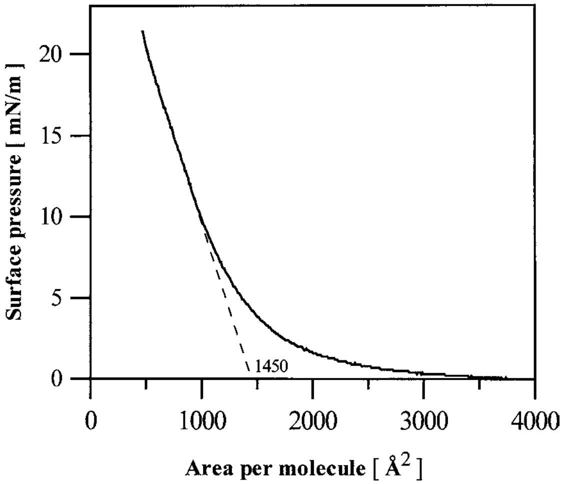

Fig. 3. Surface-pressure-molecular area isotherm of compression of the monomolecular layer formed with LHCII at the argon–water interface. The dashed line fitted to the linear part of the isotherm points into the specific molecular area of LHCII (1450 Å^2).

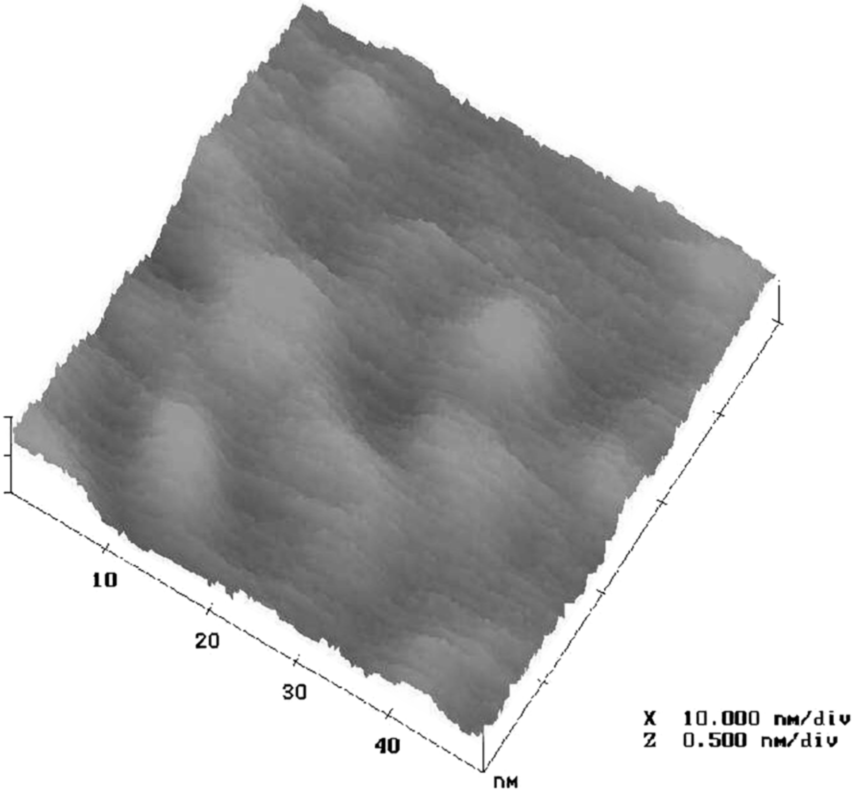

Fig. 4. Scanning force microscopy image of LHCII monolayer deposited to mica by means of Langmuir-Blodgett technique. The scan was recorded in a contact mode with NanoScope instrument from Digital Instruments at rate 3.4 Hz in cooperation with Dr. Peter Kernen from ZYOMYX Inc., Hayward California. Bulk structures visible in the topography of the Langmuir-Blodget film of LHCII correspond to the protein trimeric structures.

5. The liposome suspension is then mixed with the suspension of LHCII in the same buffer.
6. The mixture is vigorously vortexed for 5 min and then subjected to short pulses of sonication (five times for 3 s).
7. The liposome suspension is then subjected to 3-min centrifugation at 15,000*g*, in order to remove LHCII in an aggregated state in water phase that has not been incorporated to liposomes.
8. An incorporation rate can be analyzed by evaluation of chlorophyll *a* or chlorophyll *b* concentration in the liposome suspension of the known lipid content.

3.3. LHCII in Planar Bilayer Lipid Membranes

1. Planar bilayer lipid membranes (BLMs) can be formed from asolectin or egg yolk phosphatidylcholine dissolved in *n*-decane.
2. A small amount of forming solution was applied to a hole (1 mm in diameter) in a Teflon cup located in a Teflon or Plexiglas chamber.
3. The membrane separated two compartments, each of them containing a buffer called bathing solution.
4. A typical volume of these compartments is around 10 mL.
5. The solutions in both compartments have to be stirred with magnetic microstirrers during the formation of a membrane and experiments.
6. The process of membrane thinning to the stage of a bilayer can be monitored by visual inspection through a microscope and/or by measuring the electrical resistance across the membrane.
7. Reconstitution of LHCII into BLM is performed by the fusion of liposomes containing incorporated protein into a planar lipid membrane ***(9,10)***.
8. After the bilayer is formed, 50 μL of liposome suspension is added to one compartment defined as *cis*. Next, $CaCl_2$ is added to both compartments to get a final concentration of 10 m*M* Ca^{2+} (*see* **Note 4**).
9. Next, an osmotic gradient is formed between the *cis* and *trans* sides of the membrane by adding glycerol to the *cis* compartment to get to a final concentration of 0.2 *M*.
10. The process of membrane fusion and incorporation can be monitored by electrical measurements of the membrane conductivity (incorporation manifested by an increase).
11. The incorporation process is usually completed within 20 min after injection of the liposome suspension.

4. Notes

1. In order to prepare deposition solution for LHCII monolayers a suspension of the protein in the buffer has to be supplemented with 2-propanol to the final concentration of 25% (by volume) gradually. The addition of small aliquots of the alcohol and continuous stirring avoid LHCII denaturation and pigment extraction. The optimal deposition volume is between 50 μL and 100 μL. The initial surface area of deposition has to be at least 4000 $Å^2$ per single LHCII monomer in order to provide conditions for the formation of homogeneous layer. Concentration of LHCII in a deposition medium is evaluated on the basis of chlorophyll *a* or chlorophyll *b* concentration and stoichiometry of pigment binding to this complex.
2. The deposition ratio (the ratio of a surface of solid support covered by monolayer and the decrease in the surface of monolayer during deposition of the L-B film) close to 1 reflects efficient deposition of a LHCII monolayer to the support.
3. The temperature of sonication, while preparing liposomes, has to be adjusted according to a lipid component in order to maintain lipids in the liquid crystalline form (above the main phase transition).
4. Specific resistance of the BLM membrane before incorporation of LHCII has to be in the range of magnitude of 10^8 Ω cm^2 and the characteristic membrane capacitance around 0.4 μF cm^{-1}.

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19

Photosystem II Reconstitution Into Proteoliposomes

Structure–Function Characterization

Mário Fragata

Summary

This chapter discusses the photosystem II (PSII) reconstitution into proteoliposomes. In the first part of the chapter, protocols are outlined ***(1)*** for the preparation of lipid bilayer vesicles (liposomes) constituted of individual thylakoid lipids or their mixtures ***(2)*** for the preparation of PSII particles, and ***(3)*** for the incorporation of the PSII particles into the liposomes. In the second part of the chapter, methodologies are described for the structure–function characterization of the PSII–lipid complexes (proteoliposomes). This includes the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) determination of the PSII proteins, measurement of oxygen-evolving activity of PSII in the proteoliposomes, and the study of structural changes of the PSII proteins upon their incorporation into the lipid bilayers by Fourier transform infrared (FTIR) spectroscopy.

Key Words: Chloroplast lipids; infrared spectroscopy; lipid bilayer vesicles; lipid phase transitions; liposomes; oxygen evolution; photosystem II (PSII); proteoliposomes; structure–function; thylakoid membrane.

1. Introduction

The chloroplasts of higher plants and algae have three membranes (outer, inner, thylakoid) constituted of similar glyco- and phospholipids ***(1)*** for example, monogalactosyl diacylglycerol (MGDG; nonionic lipid), digalactosyl diacylglycerol (DGDG; nonionic lipid), sulfoquinovosyl diacylglycerol (SQDG; anionic lipid), phosphatidylglycerol (PG; anionic lipid), phosphatidylinositol (PI; nonionic lipid), and phosphatidylcholine ***(2–4)*** (PC; zwitterionic lipid between pH 2.0 and 10; *see* **Note 1**). Their average compositions in the outer, inner, and thylakoid membranes, as reported in **refs. *5*** and ***6***, are displayed in **Table 1**. The table shows that the inner and thylakoid membranes have similar lipid content.

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Table 1
Average Lipid Compositions (in Mol %) in the Outer, Inner, and Thylakoid Membranes of the Chloroplast of Higher Plants

	Chloroplast lipids[a]						
Chloroplast membrane	MGDG	DGDG	SQDG	PG	PC	PI	Refs.
Outer	17	39	6	10	32	5	***5***
Inner	49	30	5	8	6	1	***5***
Thylakoid	52	26	6.5	9.5	4.5	1.5	***5***
Thylakoid	54	24	9.6	7.4	4.8		***6***

[a] DGDG, digalactosyl diacylglycerol; MGDG, monogalactosyl diacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyl diacylglycerol.

On the contrary, the outer membrane has relative PC and MGDG compositions that differ considerably from the lipid content observed in the two other membranes. Specifically, a larger amount of PC in relation to MGDG is present.

A major question in the study of structure–function relations of the chloroplast lipids in photosynthesis is how to examine the functional dissimilarities of the three chloroplast membranes, i.e., the import of cytosolically synthesized proteins through the outer and inner membranes ***(7)*** or the primary processes of photosynthesis in the thylakoids ***(8)***, in the context of the similarities and dissimilarities of lipid structure and composition referred to above. Several methods are available for the study of the role of chloroplast lipids (*see* **Note 2**). In this chapter, the reconstitution of purified photosystem (PS) II particles into proteoliposomes composed of chloroplast lipids is described. This methodology is useful in the study of the thylakoid lipids effect on oxygen evolution and electron transfer, and the characterization of the lipid–protein interactions that are at the origin of protein folding or misfolding.

In the first part of this chapter, protocols are given for the incorporation of the PSII complex into lipid bilayer vesicles (proteoliposomes) constituted of individual thylakoid lipids or their mixtures. The second part of the chapter contains methodologies for the characterization of the PSII function in the proteoliposomes by measuring oxygen evolution with the Clark electrode, and the study of structural changes of the PSII proteins upon their incorporation into the lipid bilayers by Fourier transform infrared (FTIR) spectroscopy.

2. Materials

Often, it is necessary to purify the salts and lipids used in the preparation of proteoliposomes, as well as to analyze the chemical structure of the fatty acid chains of the glyco- and phospholipids. The procedures for the purification of salts are given in **Note 3**, lipids in **Note 4**, and fatty acid analysis is discussed in **Note 5**.

2.1. Preparation of Lipid Bilayer Vesicles

1. Amicon cell equipment.
2. Buffer: 20 m*M* MES (2-[*N*-morpholino]ethanesulfonic acid)-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.
3. Chromatography columns.
4. Gas chromatograph (e.g., Varian, model 3700, equipped with a Shimadzu integrator, model C-R3A).
5. HPLC equipment.
6. Lipids:
   Digalactosyl diacylglycerol (DGDG).
   Monogalactosyl diacylglycerol (MGDG).
   Sulfoquinovosyl diacylglycerol (SQDG).
   Phosphatidylcholine (PC).
   Phosphatidylglycerol (PG).
   Phosphatidylinositol (PI).
7. Millipore equipment.
8. Sepharose-4B gel (Pharmacia Fine Chemicals).
9. Sonicator (e.g., Heat Systems-Ultrasonics, Plainview, NY).

2.2. Preparation of PSII Particles

1. Barley seeds (or other adequate plant material).
2. Buffer A: 50 m*M* Tricine (*N*-tris[hydroxymethyl]methylglycine)-NaOH (pH 7.8), 400 m*M* sorbitol, 10 m*M* NaCl, and 5 m*M* $MgCl_2$.
3. Buffer B: 50 m*M* Tricine-NaOH (pH 7.8), 10 m*M* NaCl, and 5 m*M* $MgCl_2$.
4. Buffer C: 20 m*M* MES (2-[*N*-morpholino]ethanesulfonic acid)-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* MgCl2.
5. Buffer D (PSII storage buffer): 20 m*M* MES-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, 5 m*M* $MgCl_2$, and 30% (v/v) glycerol.
6. Cheesecloth tissue (Vertac™, Graphic Arts Cheesecloth, Veratec Inc., Graphic Arts Products, Walpole, MA).
7. Salts: $MgCl_2$ and NaCl.
8. Sugars: sorbitol and sucrose.
9. Triton X-l00.
10. Vermiculite and polythene trays (or other adequate material).

2.3. PSII Reconstitution into Proteoliposomes

Buffer C: 20 m*M* MES (2-[*N*-morpholino]ethanesulfonic acid)-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.

2.4. SDS-PAGE

1. SDS-PAGE equipment.
2. Resolving gel: acrylamide, ammonium persulphate, bis-acrylamide, isopropanol, SDS, TEMED (*N,N,N',N'*-Tetramethyl ethylenediamine, *N,N,N',N'*-Di-(dimethylamino) ethane, or *N,N,N',N'*-Tetramethyl-1,2-diaminomethane), Tris-HCl buffer, urea.
3. Stacking gel: acrylamide, ammonium persulphate, bis-acrylamide, SDS, TEMED, Tris-HCl buffer, urea.
4. Sample buffer: bromophenol blue, glycerol, 2-mercaptoethanol, SDS, urea.
5. Resolving gel buffer: hydrochloric acid, trizma base (Sigma Chemical Co., St. Louis, MO).
6. Stacking gel buffer: sulphuric acid, trizma base.
7. Lower reservoir buffer: hydrochloric acid, trizma base.
8. Upper reservoir buffer: boric acid, SDS, trizma base.
9. Staining solution: acetic acid, Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA), methanol.
10. Destaining solution: acetic acid, methanol.
11. A kit of protein molecular mass markers (e.g., RPN 800 kit from Amersham International plc, Buckinghamshire, UK).

2.5. Measurement of Oxygen Evolution

1. Argon or nitrogen cylinder.
2. Buffer C: 20 m*M* MES-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.
3. 2,5-Dichloro-*p*-benzoquinone (DCBQ).
4. Dithionite.

2.6. Structural Study of PSII and PSII–Lipid With FTIR Spectroscopy

1. Argon or nitrogen cylinder.
2. Buffer C: 20 m*M* MES – NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.
3. FTIR spectrophotometer.
4. Software program for the mathematical treatment of the infrared data; the use of one of the Spectra-Calc programs from Galactic Industries Corp. (Salem, NH) is recommended, but any appropriate mathematical software is acceptable.
5. Infrared spectroscopy optical windows (BaF_2 or ZnSe).

3. Methods

Subheading 3.1. outlines the protocols for the preparation of lipid bilayer vesicles, the extraction of PSII particles, and the PSII reconstitution into proteoliposomes. **Subheading 3.2.** describes procedures for SDS-PAGE, the measurement of oxygen evolution in PSII, and the structural study of the proteoliposomes by FTIR spectroscopy.

3.1. Preparation of PSII Proteoliposomes

The protocols described in **Subheadings 3.1.1.**, **3.1.2.**, and **3.1.3.** are well-known, reliable procedures for the preparation of lipid bilayer vesicles (liposomes) of about 250 Å (25 nm) diameter, the preparation of PSII particles, and the reconstitution of the PSII particles into the lipid bilayer vesicles (proteoliposomes), respectively. It is noted, however, that several other procedures for performing the same purpose are available in the literature, as well as a vast array of other methods designed for fundamental and applied research such as the many present day therapeutical purposes. **Note 6** guides the reader to some of those methodologies.

3.1.1. Preparation of Lipid Bilayer Vesicles

Before attempting to form lipid bilayer vesicles (liposomes) in aqueous media, one might consider any available information on the phase behavior of the lipids, a question that is crucial for the success of any preparation (discussed in **Note 7**). Determine which lipids are bilayer-forming or nonbilayer-forming; second, address the question of the phase transition temperatures of the lipids. The latter point is particularly important because in most cases it is not possible to obtain a liposome preparation at temperatures below the gel-to-liquid crystal phase transition temperature of the lipid. For example, lipid vesicles never form in aqueous mixtures of dipalmitoyl phosphatidylcholine (DPPC) with water at room temperature (*see* **Note 7**). In fact, the temperature of most laboratories is well below the gel-to-liquid crystal transition of DPPC of 41°C ***(14)***, thereby indicating the need for heating the water-DPPC mixture to temperatures above the 41°C limit.

The method described here is a standard procedure used for the formation of lipid bilayer vesicles constituted of either one lipid class or binary and ternary mixtures of different lipid species ***(4,15,21,22)***:

1. Solubilize 20 to 150 mg of the purified lipids (*see* **Note 4**) in diethyl ether (or another appropriate organic solvent), then dry under a current of nitrogen or argon to avoid oxidation of the lipids. Note, however, that the solubilization step can be skipped if the preparation of the bilayer vesicles is made with only one purified lipid species.
2. Disperse the dried lipids in 10 mL of a buffer medium containing 20 m*M* MES (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$ to give a lipid concentration of 10 mg/mL in the the final solution (*see* **Subheading 2.1.**).
3. Sonicate suspension in a test tube with a titanium sonicator probe (e.g., Heat Systems-Ultrasonics, Plainview, NY) for 15 min at 160 W output in a sonifier cell disrupter from Heat Systems-Ultrasonics, model W-225R, having nitrogen or argon bubbling into the solution (*see* **Note 8**).
4. Centrifuge the mixture at 100,000*g* for 1 h and concentrate the supernatant to 1 or 2 mL in a Amicon cell (or a similar equipment).

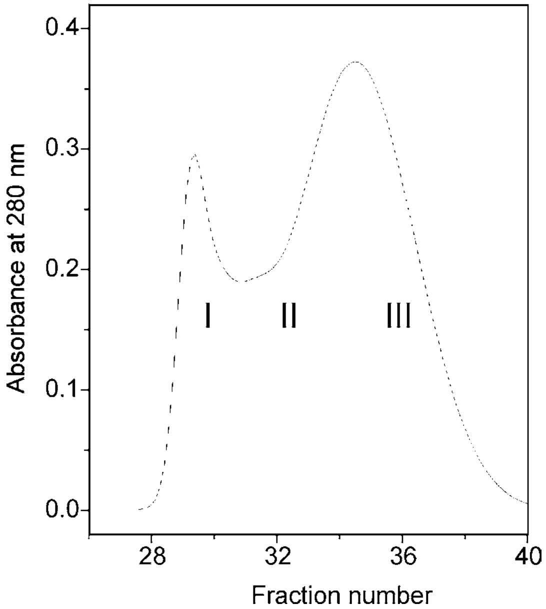

Fig. 1. Elution diagram of phosphatidylcholine vesicles prepared by ultrasonic dispersion in a buffer containing 20 m*M* MES (2-[*N*-morpholino]ethanesulfonic acid)-NaOH (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$ followed by ultracentrifugation (100,000*g*; 1 h) and gel filtration in a Sepharose-4B column. The volume of each fraction is 5 mL. I, class I liposomes constituted of large multilayered vesicles; II, class II liposomes constituted of mixtures of class I and class III; III, class III liposomes constituted of small single-layered vesicles of about 20–25 nm diameter.

5. Fractionate the lipid dispersion in a Sepharose-4B column (Pharmacia Fine Chemicals, 2.5 × 60 cm) and elute with the above described buffer (*see* **Subheading 2.1.**). Then, 5-mL fractions are collected. A typical elution diagram is provided in **Fig. 1**.
6. Only the homogeneous fractions constituted of unilamellar vesicles (class III vesicles; **Fig. 1**) are retained for the preparation of proteoliposomes.

3.1.2. Preparation of PSII Particles

Before proceeding into the description of the methods used here, it is useful to emphasize that:

1. The following protocols are designed for the extraction of PSII particles from barley (*Hordeum vulgare*) seedlings; these protocols may need to be changed if other biological materials are used to obtain the PSII particles.

2. All the steps described in this section are performed under green dim light at temperatures in the 0–4°C range.
3. All glassware, buffers and other materials must be precooled in a refrigerator before use, and maintained in containers filled with ice during the extraction procedures.
4. All biological materials (chloroplasts, thylakoid membranes, PSII particles) must always be kept in closed dark containers filled with ice, unless one chooses to work in a cold room under green dim light.

3.1.2.1. CULTIVATION OF BARLEY SEEDLINGS

The following are the steps for soaking the seeds in water.

1. Wash 200 g of barley seeds throughly, then spread inside polythene trays; add water to the trays until the seeds are immersed.
2. Cover the seeds with a layer of white tissue paper.
3. Leave the trays in darkness for 24 h at room temperature.

The following are the steps for planting the seeds on vermiculite. All the steps should take place in a growth chamber, if possible.

1. Add vermiculite to polythene trays to a height of 2 to 3 cm.
2. Spread the barley seeds (presoaked in water; **Subheading 3.1.2.1.**) on the surface of the vermiculite and sprinkle the seeds with water; the water should be spread over the whole area of the polythene tray.
3. Spread vermiculite over the seeds to a height of 1 cm in order to cover all the seeds.
4. Let the seeds germinate and grow for about 6 to 8 d at 23 ± 2°C and a light intensity of 200 ± 20 μmol m^{-2} s^{-1}.
5. After germination, sprinkle the seeds every day with about 200 mL water until the seedlings harvest.

3.1.2.2. ISOLATION OF CHLOROPLASTS AND THYLAKOID MEMBRANES

Thylakoid membranes are obtained from chloroplasts of 6- to 8-d-old-barley (*Hordeum vulgare*) seedlings according to the procedure of Berthold et al. ***(23)*** with modifications described in ***(24,25)***.

1. Cut barley leaves to 1 cm length and introduce in a precooled homogenizer jar to full volume.
2. Homogenize the barley leaf fragments (10–15 s) at 0°C in a precooled buffer medium containing 50 m*M* Tricine-NaOH (pH 7.8), 400 m*M* sorbitol, 10 m*M* NaCl, and 5 m*M* $MgCl_2$ (buffer A) (*see* **Subheading 2.2.**).

   The homogenization should take place in the dark. Cover the homogenizer jar with a black cloth during operation.
3. Filter the homogenized slurry through eight layers of cheesecloth tissue, then centrifuge the filtrate at 1000*g* for 5 min at 4°C.
4. The pellet obtained in **step 2** (chloroplasts) is dispersed in buffer A (*see* **Subheading 2.2.**) to a total volume of 200–400 mL using a brush and a Vortex mixer; then, the dispersion is centrifuged at 1000*g* for 5 min at 4°C.

5. Suspend the chloroplast pellet in a buffer containing 50 m*M* Tricine-NaOH (pH 7.8), 10 m*M* NaCl, and 5 m*M* $MgCl_2$ (buffer B) (*see* **Subheading 2.2.**) using brush and Vortex mixer; centrifuge at 1000*g* for 5 min at 4°C.
6. Suspend the pellet obtained in **step 4** (thylakoid membranes) in a minimal volume of a precooled buffer medium containing 20 m*M* MES (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$ (buffer C) (*see* **Subheading 2.2.**) using brush, Vortex mixer and a teflon homogenizer; this step might be followed by a centrifugation at 1000*g* for 5 min at 4°C.
7. Suspend the pellet of thylakoid membranes obtained in **step 5** in buffer C (*see* **Subheading 2.2.**) to give a final chlorophyll (Chl) concentration of 2 mg/mL, and store at approx –80°C until use.
8. The Chl concentration is estimated using 80% (v/v) acetone solutions according to the method of Arnon ***(26)*** (*see* **Note 9**).

3.1.2.3. ISOLATION OF PSII PARTICLES

1. Bring thylakoid membranes kept at –80°C (*see* **Subheading 3.1.2.2.**) to 0°C in a container filled with ice.
2. Estimate the chlorophyll concentration in the thylakoid samples according to the procedure in **Subheading 3.1.2.2.**
3. Assay the oxygen-evolving activity of the thylakoid membranes prior to the isolation of the PSII particles.
4. Using a Vortex, mix 1 mL of the thylakoid membranes suspension (2 mg Chl/mL) with 0.5 mL of buffer C (*see* **Subheading 2.2.**).
5. With very gentle shaking, add dropwise 0.5 mL of 8% Triton X-l00 to the mixture obtained in **step 4** to give a final Chl concentration of 1 mg/mL; the final Triton X-100/Chl ratio should be 20/1.

   During the addition of the Triton X-100 solution the use of a Vortex, or any other mechanical shaking device, should be avoided, this step must be done slowly, but as fast as possible, and the use of gloves is mandatory to avoid any skin contact with the Triton X-100 solution.
6. Incubate the suspension obtained in **step 5** in the dark for 20 min at 4°C, then centrifuge at 1000*g* for 3 min at 4°C to precipitate the unbroken thylakoids.
7. Transfer supernatant of **step 6** to one or several prechilled centrifuge tubes and centrifuge immediately at 29,000*g* for 30 min at 4°C.
8. Discard the supernatant of each centrifuge tube of **step 7** (containing Triton X-100), and add 1 mL of buffer C (*see* **Subheading 2.2.**) to the pellet of each of the tubes, followed by dispersing the pellets using a brush.
9. Add 10–15 mL of buffer C (*see* **Subheading 2.2.**) to each one of the centrifuge tubes in **step 8**, and centrifuge immediately at 29,000*g* for 30 min at 4°C; in this step, the residual Triton X-100 will be removed.
10. Pool the pellets of all tubes used in **step 9** (PSII particles) together upon suspension in small volumes of buffer D (*see* **Subheading 2.2.**) to give a final volume of 1 mL.
11. Immediately after **step 10**, or as soon as possible, determine the characteristics of the PSII particles just prepared by measuring their chlorophyll content, the oxy-

gen-evolving activity, and the polypeptide composition with SDS-PAGE. It is worth noting that the electrophoresis test is particularly important to check whether the PSII particles are contaminated with PSI proteins.

12. Finally, the PSII particles are stored at –80°C until use.

3.1.3. PSII Reconstitution Into Proteoliposomes

It is important to note that all the following steps are performed in green dim light and at temperatures of approx 4°C; work either in a cold room in green dim light, or maintain all buffers and other materials in dark containers filled with ice.

To prepare the PSII–lipid membranes follow the following steps ***(4,24,25)***:

1. Centrifuge solubilized particles at 29,000*g* for 30 min at 4°C, then suspend in 20 m*M* MES (pH 6.5), 400 m*M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$ (buffer C) (*see* **Subheading 2.2.**); this step is repeated once using buffer C as the suspension medium.
2. Gently mix the washed PSII particles obtained in **step 1** in a vortex with the lipid vesicles (*see* **Subheading 3.1.1.**); the lipid:chlorophyll ratio is maintained at 20/1 (w:w) throughout ***(4)***.
3. The salts can be added at this stage to study the effect of ions on the activity of the PSII–lipid complex.
4. Incubate the protein–lipid mixtures in darkness at 0°C for 20 min.
5. Immediately after the incubation step, use an aliquot of the preparation for oxygen evolution determination according to the procedure outlined below (*see* **Subheading 3.2.2.**).
6. Measure their oxygen evolution activity.

3.2. Characterization of PSII Proteoliposomes

The protocols described here include first standard procedures of SDS-PAGE, and the measurement of oxygen evolution with a Clark-type electrode (**Subheadings 3.2.1.** and **3.2.2.**). Next, **Subheading 3.2.3.** outlines the essentials of the FTIR spectroscopy methodology, which has been successfully used in a large number of studies. For example, the examination of structural changes in the photosynthetic membrane induced by a variety of effects such as ultraviolet (UV)-B radiation ***(27)*** and heat stress ***(28)***. The examples discussed are limited to the analysis of the "spectral window" between 1700 and 1480 cm^{-1} (**Figs. 2–4**) where most biolipids have no significant infrared absorption. Procedures are described for the preparation of samples for FTIR spectroscopy, the measurement of infrared spectra, and the analysis of infrared data.

3.2.1. SDS-PAGE

The polypeptide composition of the PSII particles is analyzed by SDS-PAGE to determine whether they are contaminated with PSI proteins ***(8,30)***.

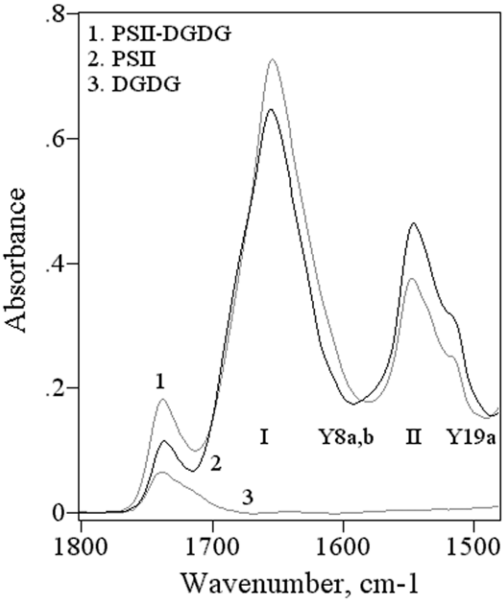

Fig. 2. Infrared spectra of photosystem II (PSII), digalactosyl diacylglycerol (DGDG) and the PSII–DGDG complex between 1800 and 1480 cm^{-1}. Abbreviations: I, amide I region (1700–1630 cm^{-1}); II, amide II region (1580–1520 cm^{-1}); Y8a,b and Y19a, regions of the 8a and 8b (1630-1580 cm^{-1}) splitting components of the fundamental mode no. 8 and the high-frequency splitting component 19a (1520–1500 cm^{-1}) of the phenol ring according to Wilson *(39)*. Note that in the *"spectoscopic window"* from 1700 to 1480 cm^{-1}, DGDG has negligible absorbance.

First, it is mentioned that the protocols outlined hereunder (preparation of the gels for SDS-PAGE as well as many other experimental details) are taken from the procedures described in the work of Chua *(29)*. Consult before proceeding further in this section. In addition, it is specially important to note the following:

1. All steps described below are performed at room temperature.
2. SDS and TEMED may be stored at room temperature.
3. Always prepare fresh solutions of urea and ammonium persulfate.
4. While handling acrylamide (monomeric product), use a mouth-and-nose mask and a pair of gloves; this precaution is not necessary upon polymerization of the acrylamide.

The standard proteins and PSII polypeptides are resolved on a linear gradient gel with the separating gel containing 15% acrylamide and 6 *M* urea, and the stacking gel having 5% acrylamide and 6 *M* urea. Before loading into the gel slots, the PSII samples are first solubilized with SDS. This is followed by a

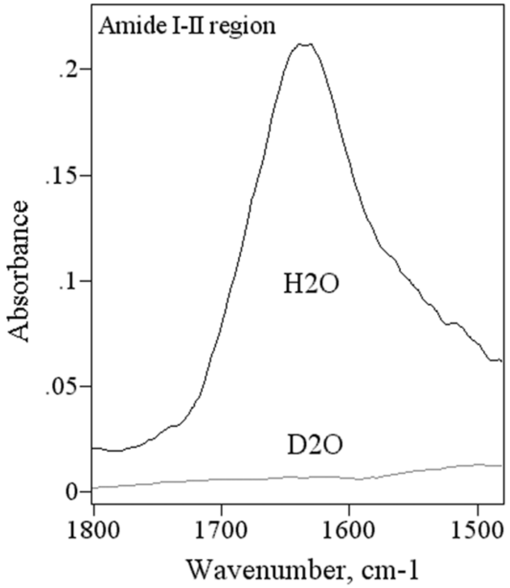

Fig. 3. Infrared absorbance of H_2O and D_2O between 1800 and 1480 cm^{-1}, in the spectral regions of the amide I, amide II, and the splitting components of the fundamental modes no. 8 and no. 19 of the phenol ring **(Fig. 2)**.

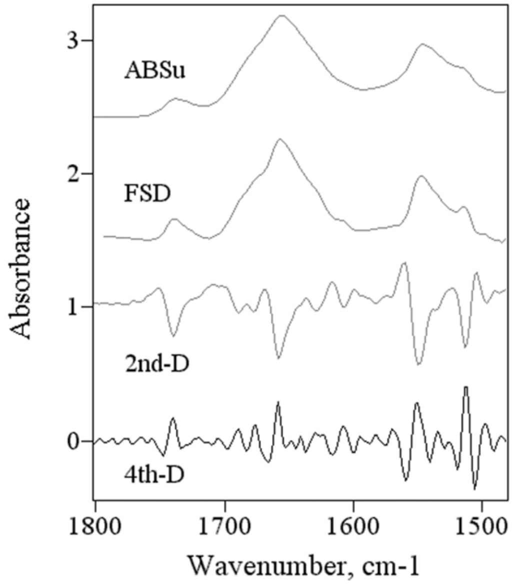

Fig. 4. Resolution enhancement of a untreated infrared spectrum of photosystem II (ABSu) using the Fourier self-deconvolution (FSD), second derivative (2nd-D) and fourth (4th-D) derivative methods. The calculations were performed with the "mathematical tool" of the Spectra-Calc program.

centrifugation at 13,600*g* for 5 min to remove the unsolubilized membranes. Upon electrophoresis, the gels are stained for 3 h with a solution containing 50% methanol, 10% acetic acid, and 0.125% R-250 Coomassie brilliant blue (Bio-Rad Laboratories, Richmond, CA). Gel destaining is done in solutions containing 60% water, 30% methanol, and 10% acetic acid. To estimate the molecular mass of the proteins, a plot of log molecular mass vs the migrated distance (in centimeters) is used for the unknown proteins and standard protein markers. The molecular masses of the markers in the RPN 800 kit from Amersham International plc (Buckinghamshire, UK) are 15, 25, 30, 35, 50, 75, 105, 160, and 250 kDa.

The PSII polypeptide subunits usually detected in the electrophoresis gel are the 16-, 23- and 33-kDa extrinsic proteins, the S protein, the α-cyt b559, the proximal antenna proteins (CP43 and CP47), and the D1 and D2 proteins in the reaction center (34 and 32 kDa, respectively) ***(8)***.

3.2.2. Measurement of Oxygen Evolution

Measurement of electron transport through PSII estimated as oxygen evolution in the PSII particles and the PSII–lipid complex is performed at 25°C with a Clark-type electrode using, for example, the equipment from Hansatech D.W. Oxygen Electrode Unit, King's Lynn, Norfolk, UK (*see* **Note 10**).

The reaction medium (total volume: 2 mL) contains the following:

1. 1960 μL of the measurement buffer (buffer C; *see* **Subheading 2.2.**).
2. 20 μL of 350 μ*M* DCBQ (2,5-dichloro-*p*-benzoquinone) as exogenous electron acceptor at the QA site ***(8)***, and
3. 20 μL samples of PSII particles or PSII–lipid complex (final concentration: 12.5 μg Chl/mL).

The PSII and PSII–lipidI preparations are then illuminated with white light at saturating light intensity. Note that usually the universal unit used for expressing oxygen-evolving activity is μmol O_2/mg Chl/h.

For the measurement of the pH effect on oxygen evolution, adjust the pH of the buffer media in the pH 3.0–10 range with HCl or NaOH. This practice is useful to avoid changes of chemical composition of the reaction media when one uses three different buffer systems to cover the pH 3.0–10 range.

3.2.3. Structural Study of PSII and PSII–Lipid With FTIR Spectroscopy

FTIR spectroscopy is widely used in structural studies of proteins ***(31)***. Among those studies, many examined the structure–function relations in the photosynthetic membrane ***(32–35)***. Although the useful infrared range spans from about 4000 to 600 cm^{-1}, most works are performed between 1700 and 1480 cm^{-1}, i.e., the amide I and amide II regions. The reason for this is that these spectral regions contain a set of vibrational modes that are related to several periodic

Table 2
Wavenumbers and Vibrational Modes of the Major Spectral Bands Between 1760 And 1500 cm^{-1} in the Infrared Spectrum of Photosystem II With Indication of Protein Periodic Structures in the Amide I and Amide II Regions[a]

Spectral region	Wavenumber (cm^{-1})	Vibrational modes and protein periodic structures[b]
	Vibrational modes	
Amides I[c], II[d]	1627	$\delta_a NH_3^+$ or δNH_2
1572, 1564	$\nu_a COO^-$	
1550, 1531	$\delta_s NH_3^+$	
Tyrosine	**1620–1580**	
	~1608	$\nu 8a$ ring[e]
	~1590	$\nu 8b$ ring[e]
	1520–1500	$\nu 19a$ ring (~1508 cm^{-1}) + deformation[e]
Chl + lipids	**1760–1692**	
	~1739	νCO (*sn*1 or *sn*2 ester CO) of lipids
	~1737	νCO (ester CO) of chlorophylls
	~1693	νCO (free keto CO) of chlorophylls
	~1662	Bound keto CO of chlorophylls
	Protein periodic structures	
Amide I	**1696–1620**	
	1696–1665	Turns (e.g., β-turns), antiparallel β-sheet (~1693 cm^{-1})
	1658–1654	α-helix + some random coil structures
	1648–1641	Random structures, loops
	1640–1620	β-sheet (~1636 cm^{-1}), extended chain (β-strand: ~1626 cm^{-1})
Amide II	**1580–1520**	
	~1545	Some α-helical conformations

[a] Data from Arrondo et al. *(31)*, Gabashvili et al. *(35)*, Ségui et al. *(27)*, and literature cited therein.

[b] Chl, chlorophyll; δ, bending; δ_α, asymmetric bending; δ_s, symmetric bending; ν, stretching; ν_a, asymmetric stretching.

[c] Amide I: In-plane C = O stretching vibrations weakly coupled with CN stretching and CCN deformation.

[d] Amide II: In-plane NH bending strongly coupled with CN stretching.

[e] Wilson et al. *(39)*.

structures found in many proteins. In addition, the amide I and amide II regions are very sensitive to conformational changes in the proteins secondary structures such as those caused by UV light *(27)* or thermal effects *(28,34,36)*.

Table 2 collects wavenumbers and vibrational modes of major bands between approx 1780 and 1500 cm^{-1} in the infrared spectra of PSII, and provides information on the protein periodic structures deduced from the spectral data *(27,31,35–39)*.

1. The region from 1780 to 1700 cm^{-1} is characterized by vibrational modes originating in the lipids and chlorophylls.
2. The region from 1700 to 1600 cm^{-1} (amide I) is associated with an in-plane C = O stretching vibration (approx 80%) weakly coupled with some contributions from CN stretching and CCN deformation.
3. The region from 1580 to 1520 cm^{-1} (amide II) is assigned to an in-plane NH bending strongly coupled with CN stretching.
4. The spectral bands at about 1608, 1590, and 1508 cm^{-1} originate in the vibrational modes of the phenol ring, e.g., in tyrosine.

The question of the "spectral window" between 1700 and 1480 cm^{-1} in lipid–protein studies is discussed first, then methods are outlined for the preparation of samples for FTIR spectroscopy, the measurement of infrared spectra, and the analysis of infrared data.

3.2.3.1. "Spectral Window" Between 1700 and 1480 cm^{-1} in Infrared Spectra of Biolipids

It is interesting to note that in the study of lipid–protein interactions one takes advantage of a useful "spectral window" between 1700 and 1480 cm^{-1} where most lipids do not absorb infrared radiation ***(35,40)***. This is clearly seen in **Fig. 2** for the interaction between PSII and the galactolipid digalactosyl diacylglycerol (DGDG). That is, from about 1700 to 1480 cm^{-1} the DGDG absorbance is negligible compared to the large absorbance of infrared light by the PSII and PSII–DGDG complexes. This means that within the 1700–1480 cm^{-1} range any lipid-induced change of the infrared spectra of the PSII–lipid complex is not affected by the lipid absorbance observed either at wavenumbers greater than 1700 cm^{-1} or smaller than 1480 cm^{-1}.

3.2.3.2. Preparation of Samples for FTIR Spectroscopy

Infrared investigations of protein–lipid interaction are carried out in deuterium oxide (D_2O) to overcome the strong overlapping effect of the H_2O absorption in the amide I and amide II regions (*see* **Fig. 3**). The protocols given below, ***(41)***, outline a modified procedure for preparing liposomes and proteoliposomes in D_2O, and the steps for preparation of samples for layering on BaF_2 or ZnSe plates.

3.2.3.2.1. Preparation of Liposomes and Proteoliposomes in D_2O

1. The lipid bilayer vesicles (liposomes) are prepared according to the protocols described in **Subheading 3.1.1.** except for replacement of the water solvent with D_2O.
2. For preparing the PSII–lipid membranes (proteoliposomes), wash the PSII particles obtained in **Subheading 3.1.2.** in D_2O by centrigugation at 28,700*g* for 7 min at 4°C, then complex with the liposomes obtained in step (a) (*see* **Subheading 3.1.3.**).

3.2.3.2.2. Preparation of Samples for Layering on BaF_2 or ZnSe Plates

1. Centifuge aliquots of the PSII or PSII–lipid samples (1.5 mg ChL/mL) obtained as described in **step 1** at 28,700*g* for 7 min at 4°C.
2. Suspend the pellet in 3 mL D_2O and centrifuge a second time (28,700*g*; 7 min; 4°C).
3. Suspend the final pellet again in a D_2O volume sufficient to make a solution with a chlorophyll concentration of 0.333 mg/mL.
4. Immediately layer an aliquot of the solution on 25 mm diameter BaF_2 or ZnSe plates that are stored in darkness on filter paper placed on ice in a controlled temperature chamber (0°C).
5. Dehydrate for about 90 min under a current of nitrogen.

In the above conditions any 1H/2H exchange is minimized, thereby assuring that the results are not influenced significantly by the presence of D_2O molecules in the PSII samples.

3.2.3.3. MEASUREMENT OF INFRARED SPECTRA

The infrared absorbance measurements are made in a Nicolet FTIR spectrophotometer, model 420, or another appropiate instrument. In the Nicolet FTIR instrument, 100 interferograms are collected and co-added using Omnic software facility. The infrared spectra are obtained upon subtraction of the spectrum of the BaF_2 or ZnSe plates used for deposition of the samples. The spectral resolution is between 1 and 2 cm^{-1}.

3.2.3.4. ANALYSIS OF INFRARED DATA

The FTIR spectra are processed using the GRAMS/386 Spectra-Calc program, version 1.06A, from Galactic Industries Corp. (Salem, NH), or another appropriate software. Hereunder. information is provided for the baseline correction, the mathematical analysis of FTIR spectra, the determination of the contents in protein periodic structures, and the calculation of difference spectra.

1. Baseline Correction. Prior to data processing, or before any mathematical treatment of the FTIR spectra, it is important to perform a baseline correction of the original spectra between about 4000 and 600 cm^{-1} using the “baseline correction tool” of the Spectra-Calc program.
2. Mathematical Analysis of FTIR Spectra. Identification of the band maxima frequencies (or wavenumbers) is first done on inspection of the FTIR absorbance spectra. However, the amide I and amide II envelope is usually rather featureless (PSII and PSII–DGDG spectra in **Fig. 2**), rendering the analysis of the infrared spectra difficult or not worthy. To cope with this disadvantage refer to mathematical methods of “resolution enhancement”. For example, the “mathematical tool” of the Spectra-Calc program offers the possibility of performing various types of resolution enhancement: the Fourier self-deconvolution (FSD), the second and fourth derivatives, and curve-fitting analyses using Gaussian, Lorentzian, and Voigt functions.

a. Fourier Self-Deconvolution (FSD). In this method the effective spectral bandwidth is synthetically narrowed. As apodization filters, FSD uses either the Bessel or the Boxcar function, although in most instances the Bessel function is preferred. Upon application of this method it becomes easier to identify the principal bands under a much larger multiband envelope with overlapping individual band features. This is seen in **Fig. 4**, which displays a typical untreated absorbance spectrum of PSII together with its FSD spectrum.
b. Second and Fourth Derivatives. The "differentiate tool" of the Spectra-Calc program provides the first and second derivatives using the Savitsky-Golay convolution method. To obtain the fourth derivative, one uses the second derivative spectrum as the starting data for the calculation. Now, it is important for the good interpretation of the data that a "band maximum in the original spectrum" (and the FSD spectrum) is displayed as a "band minimum in the second derivative spectrum" and as a "band maximum in the fourth derivative spectrum." This is clearly seen in the amide I–II spectrum of PSII displayed in **Fig. 4**. As an example, one may wish to verify in **Fig. 4** that the α-helix band maximum (**Table 2**) seen in the original (untreated) spectrum at approx 1655.7 cm^{-1}, is displayed at 1657.7 cm^{-1} in the FSD, second derivative and fourth derivative spectra, that is, a displacement of about 2 cm^{-1} to a higher wavenumber. In most instances, the 1657.7 cm^{-1} wavenumber, is taken as a better estimation of α-helix band maxima.
c. Curve-Fitting Analyses. The "curve-fitting tool" of the Spectra-Calc program automatically calculates the best fit of Gaussian, Lorentzian, or Voigt bands that make up a complex set of overlapping peaks. The program works first on a guess at the number of overlapping bands present within the multiband envelope, their peak positions, their peak widths, and the peak types (e.g., Gaussian, Lorentzian, or their mixtures). Then, it iterates to locate a combination of band heights, positions, and widths that best fit the experimental data. It is here that the data obtained with the FSD, second derivative and fourth derivative calculations (see above) is extremely important. In fact, all the calculations suggested in this subsection should be performed sequentially, and followed by comparisons with data available in the literature. The final result of the "curve-fitting analysis" is a set of synthetized spectral bands that represent the several periodic substructures that make up the molecular, or supra-molecular, structure of the protein or protein complexes under study (**Table 2**).

3.2.3.5. DETERMINATION OF THE CONTENTS IN PROTEIN PERIODIC STRUCTURES

The procedure for determining the contents in protein periodic structures is quite simple. First, the total area of the amide I–II envelope surface from about 1700 to 1500 cm^{-1} is determined using the "integration tool" of the Spectra-Calc program. Second, the individual areas of the different spectral bands obtained in the "curve-fitting analysis" described above are well determined with the "integration tool." Third, the percentages of α-helix, β-sheet, β-strands or any of the other structures are found by computing the ratio of the individual band areas in relation to the total area of the amide I–II surface ***(40)***.

To identify a given protein periodic structrure (α-helix, β-sheet, β-strand, etc.) with a specific spectral band use the information in **Table 2**, or data given elsewhere in the literature.

3.2.3.6. CALCULATION OF DIFFERENCE SPECTRA

To obtain the difference spectra, i.e., the [PSII–lipid]-minus-[PSII] spectra, the methods developed in the literature ***(35,42,43)*** are applied. First, the areas under the spectral envelope in the 1700-1500 cm^{-1} region, i.e., amides I and II, for the PSII and the PSII–lipid spectra are determined using the "integration tool" of the Spectra-Calc program. Second, the PSII and the PSII–lipid areas are normalized according to the expression $A_n = kA_o$, where A_o is the integral of the observed surface under the band envelope calculated with the Spectra-Calc Program, k is a scale factor that normalizes A_o to 100, and A_n is the normalized area, i.e., 100. Third, the [PSII–lipid]-minus-[PSII] subtraction of the "normalized spectra" is performed.

4. Notes

1. Phosphatidylcholine in the Thylakoid Membrane. Phosphatidylcholine is one of the components of the outer membrane of the chloroplast ***(2)***. However, its presence in the thylakoids is still a matter of controversy ***(3)***. Notwithstanding, it is interesting to remark that the oxygen evolving activity of purified PSII particles is enhanced after incorporation into liposomes constituted of phosphatidylcholine ***(4)***.
2. Methodologies for Structure–function Studies of Chloroplast Lipids. Most methodologies available for the study of the chloroplast lipids role in structure–function relations are discussed in **ref. *9***. Structure–function. Revisions were presented in **ref. *10***. In short, the major methods used presently are: isolated proteins reconstituted into proteoliposomes, membranes with lipids inactivated by chemical modification or by immunological methods, membranes selectively depleted of lipids by chemical treatment, and mutants with genetically induced changes in lipid composition.
3. Salts Purification. Commercially available inorganic salts may be contaminated with organic impuritites that usually give rise to changes in the surfaces properties of native biological membranes and artificial lipid bilayer vesicles (liposomes) ***(11)***. The membrane properties affected by the presence of organic impurities in inorganic salts are, among other possible interferences, the polarity of the lipid-water interface and the evolution of interfacial reaction kinetics ***(11)***, and the lipid–protein interactions. The following procedure for purification of the salts is recommended. First, the salts are washed successively with three different organic solvents (methanol, benzene, chloroform) to remove the organic impurities. Second, the purified salts are dried at 150–160°C for 24 h to remove any traces of adsorbed

solvent. Third, the degree of purification of the salts obtained by this procedure is checked by surface tension experiments.

The surface tension data of the purified salts dissolved in water (demineralized; distilled) might be in the range of values published in the literature.

4. Lipids Purification. The routine purification procedure used takes place in a HPLC (Water Associates, Milford, MA) equipped with two 510 pumps, an automatic gradient controller (model G80), a Rheodyne injector (model 7126) (Cotati, CA), with a 1-mL loop and a programmable ultraviolet-visible detector set at 205 nm ***(12,13)***. The lipid separation is performed on a silica cation-exchange column operated in isocratic mode. All solvents (HPLC grade) used in the mobile phase are filtered through a membrane of 0.2-µm pore size (Millipore, Bedford, MA), and degassed under vacuum prior to their introduction into the HPLC system. The mobile phase is a *n*-hexane:isopropyl alcohol:water mixture, 70/30/2 (v:v:v). The flow rate is 10 mL/min at 2400 PSI. The samples are dried in a stream of nitrogen, then dissolved in the elution mixture to give a final concentration of 5 mg/mL, followed by injection via the Rheodyne rotatory injector. The purified lipids are thereupon dissolved in chloroform and kept in a deep-freezer until use.
5. Fatty-Acid Chains Analyses. Analyses of the fatty acid chains of the lipids ***(12,13)*** are done by gas chromatography of methyl esters formed from methanolysis of the lipid (0.1 mg) with 1 mL of BF3/MeOH 14% wt/vol (Pierce Chemical Co.). The mixture is heated 15 min at 100°C followed by cooling for approx 10 min; then *n*-hexane and bidistilled water are added to separate the fatty-acid methyl esters dried by treatment with anhydrous Na_2SO_4 followed by a current of nitrogen. The residue is dissolved in 50 µL of *n*-hexane. The fatty-acid analysis is carried out in a Varian gas chromatograph (model 3700), equipped with a Shimadzu integrator (model C-R3A), using nitrogen as the carrier gas ***(12,13)***.
6. Other Methods for Preparation and Use of Liposomes. The evolution of the liposome methodology has been in constant progress since its early days ***(14)***. In fact, from their initial purpose as useful models of biological molecules ***(15,16)***, the liposomes became an indispensable ingredient in the study of lipid–protein interactions (proteoliposomes), and a powerful tool as drug transport vehicles in vivo for therapeutical uses such as the delivery of anticancer agents to malignant tumors ***(17)***. Papers are regularly published on the formation of small size liposomes of approx 250 Å (0.025 µm) diameter for new biochemical and physical-chemical studies ***(17)***, and the preparation of large unilamellar vesicles (LUV or "giant liposomes") ranging from 10 to 500 µm diameter ***(18)***.
7. Phase Behavior of the Thylakoid Lipids. The phase behavior of a lipid species in its mixtures with other lipids and water is well described by the phase diagram of the lipid. It is noted, that a notable account of available phase diagrams and other related questions is given in the excellent review in **ref. *19***. Among the many other works that were published on these matters, those in **refs. *2*** and ***20*** are directly related to the chloroplast lipids. For practical purposes, there are two main reasons that render useful the consultation of the phase diagram of the lipid used in the preparation of liposomes or proteoliposomes. First, identify whether the lipid in question is a "bilayer-forming" or a "nonbilayer-forming" species; in the latter case, use

binary or ternay mixtures of lipids. Second, identify the gel-to-liquid crystal transition temperature of the lipid to know whether the formation of liposomes takes place at room temperature ***(14,19)***. It is important to emphasize that the preparation of lipid vesicles should always be performed above the phase transition temperature of the lipid species being used. This precaution is usually unnecessary with native lipids that have transition temperatures several degrees below 0°C, but is essential if the vesicles are prepared with a synthetic lipid such as dipalmitoyl phosphatidylcholine whose transition temperature is around 41°C ***(14)***, well above the room temperature of most laboratories. Detailed information on the phase behavior and hydration of most phospholipids, e.g., phosaphatidylcholine and phosphatidylglycerol, has been described in **ref. *19***.

8. Sonication in a Capped Tube. The sonication step can be also performed, whenever possible, in a special capped tube obtained from Heat Systems-Ultrasonics (Plainview, NY). The use of the capped tube avoids contamination of the lipid preparations with titanium particles from most sonicator probes.
9. Estimation of Chlorophyll Concentration. The chlorophyll concentrations of chlorplasts, thylakoid membranes, or PSII particles is estimated using 80% acetone solutions according to the procedure in **ref. *26***. In short, an aliquot of volume V_s of the sample added to a given volume of 80% acetone, V_a. The mixture is first vortexed, then centrifuged for 5 min in a IEC clinical centrifuge (or another similar model). The supernatant is carefully transferred to test tubes paying particular attention of not disturbing the centrifugation pellet. Then, the absorbance (A) of the solutions is measured in a spectrophotometer at 645 and 663 nm. Finally, the chlorophyll concentration in mg/mL is calculated using the expression

$$[\mathrm{Chl}] = [(20.2 \times A_{645} + 9.02 \times A_{663}) \times V_a] / V_s$$

   where A_{645} and A_{663} are the absorbances at 645 and 663 nm, respectively.
10. The Oxygen Electrode. In general, the Hansatech Company (http://www.hansatech-instruments.com) furnishes detailed theoretical and experimental documentation with their equipment. In addition, a copy of the book The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis by Professor David A. Walker can be obtained from Hansatech.

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Extraction of the Functional Manganese and Calcium From Photosystem II

Joel Freeman, Garth Hendry, and Tom Wydrzynski

Summary

Manganese (Mn) and calcium (Ca) are both metal cofactors required for photosynthetic oxygen evolution ***(1)***. The functional roles for these ions in the O_2-evolving reactions are not completely known. They are studied by comparative spectroscopic measurements between intact and metal-depleted samples. In this chapter, we outline three experimental procedures used for the various removal of Mn and Ca from photosystem (PS) II-containing (i.e,. O_2-evolving) preparations: the complete Mn extraction using a strong alkaline Ches buffer (pH 9.4)/$MgCl_2$ wash, partial Mn extraction using a mild hydroxylamine (pH 6.8) wash, and specific Ca extraction through a low pH/citrate (pH 3) wash. The O_2 evolution activities (measured by a Clarke-type oxygen electrode), protein composition (determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis), and the relative Mn and Ca content (measured by atomic absorption spectroscopy) are reported for each extraction procedure.

Key Words: Photosystem II; oxygen-evolving complex; manganese; calcium; photoactivation; extrinsic proteins.

1. Introduction

The photosynthetic oxidation of two water molecules into O_2, four hydrogen ions and four electrons occurs in photosystem (PS) II at an inorganic Mn_4Ca core, which, in combination with its ligating protein scaffold, is called the oxygen-evolving complex (OEC). The reaction sequence proceeds via a series of four light-driven, one-electron oxidation steps that are defined by the S_n states (where $n = 0, 1, 2, 3$, or 4). Because manganese (Mn) ions can have a number of stable oxidation states,for example, Mn(II), Mn(III), and Mn(IV), the Mn_4Ca core is considered to function as a charge accumulating unit (or battery) that upon being charged to the most oxidized S_4 state, reacts with water to produce O_2 and

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the starting S_0 state. Although low resolution (3.7–3.8 Å) crystal structures of PSII have recently appeared in the literature ***(2,3)***, the organization of the Mn_4Ca core and the mechanism of O_2 evolution are still relatively unknown. Much of what is known about the OEC has come from spectroscopic studies of PSII preparations using electron paramagnetic resonance (EPR), Fourier transform infrared (FTIR), mass spectrometric, optical, and fluorescence techniques. For these studies, the Mn and calcium (Ca) are extracted by various methods and reconstituted to evaluate the chemical properties of the catalytic site. In this chapter we report three basic procedures for variously extracting the Mn and Ca from photosystem II that are commonly used in the spectroscopic studies.

2. Materials

1. PSII-enriched membrane preparation.
2. Deionised (Milli Q) water for making solutions.
3. Acid-washed glassware.
4. Nylon cloth (~20 µ mesh).
5. Camel's hair brush (for sample resuspension).
6. Centrifuge equipment (e.g., Sorval RC5C).
7. Chelex 100 Resin (100–200 mesh).
8. Standard buffer: 25 m*M* MES-NaOH, pH 6.5, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 400 m*M* sucrose at 4°C.
9. Low MES standard buffer: 0.25 m*M* MES-NaOH, pH 6.5, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 400 m*M* sucrose at 4°C.
10. High MES standard buffer: 50 m*M* MES-NaOH, pH 6.5, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 400 m*M* sucrose at 4°C.
11. Wash buffer 1 to completely extract Mn: 20 m*M* CHES-NaOH, pH 9.4, 200 m*M* $MgCl_2$ at room temperature.
12. Wash buffer 2 to partially extract Mn: standard buffer plus 1.5 m*M* hydroxylamine at 4°C.
13. Wash buffer 3 to extract functional Ca: 200 m*M* citrate, pH 3.0, 15 m*M* NaCl, 400 m*M* sucrose at 4°C.
14. Assay buffer: standard buffer containing 1 m*M* $K_3Fe(CN)_6$ and 0.2 m*M* PPBQ (*p*-phenylbenzoquinone); a 20 m*M* stock solution of PPBQ is prepared in dimethylsulfoxide (DMSO) at 25°C.
15. 80% acetone in 1 m*M* phosphate buffer, pH 7.0.
16. Clark oxygen electrode equipment (e.g., Hansatech system with 200 W projector light source and 25°C circulating water bath).
17. Atomic absorption equipment (e.g., Varian Spectra A20).
18. Concentrated nitric and concentrated perchloric acids.
19. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment (e.g., Biorad system).
20. Urea.
21. Ethylenediaminetetraacetic acid (EDTA).

22. Ethyleneglycoltetraacetic acid (EGTA).
23. Sonicator bath.

3. Methods

The methods described below outline procedures for the complete extraction of Mn from PSII; the partial extraction of Mn; and the specific extraction of the functional Ca. Protocols for characterization of the samples by O_2 evolution activity, protein composition, and metal content are included.

3.1. General Methods

3.1.1. PSII Preparation for Ca and Mn Extraction

1. Prepare PSII-enriched membrane samples according to the method of Berthold et al. ***(4)***.
2. Isolate thylakoid membranes from market spinach by grinding about 400 g of deveined, washed leaves in 800 mL of the standard buffer in a Waring blender for 10 s at 4°C in the dark or under a weak green safelight. (The homogenization may be carried out in four, separate 100 g/200 mL batches.)
3. Filter the combined homogenate through two layers of cheesecloth and two layers of nylon cloth (20 µ mesh) and centrifuge for 5 min at 4000*g* using a Sorval GSA rotor at 4°C. 4. Resuspend the pellet containing the thylakoids (*see* **Note 1**) in 400 mL of the standard buffer and centrifuge again at the same speed.
4. Resuspend the washed pellet in a small volume of the standard buffer to a concentration of 2.5 mg chlorophyll (Chl)/mL (see below for Chl determination) and allow to sit for ≥ 15 min in the dark on ice (4°C).
5. Dilute the total volume (V) of the thylakoid suspension with a V/4 volume of 25% (v/v) Triton X-100 detergent solution added slowly (dropwise with gentle stirring over several minutes) to solubilize the stromal membranes. The 25% Triton X-100 solution is made in 20 m*M* MES-NaOH, pH 6.5. The final detergent concentration during solubilization should be 5% at a sample concentration of 2 mg Chl/mL.
6. Gently stir the thylakoid/Triton X-100 solution for 20 min in the dark on ice (4°C) before centrifuging for 1 min at 5000*g* using eight centrifuge tubes in a Sorval SS-34 rotor at 4°C in order to remove starch and unsolubilized material. (This step may be repeated if there is a lot of starch in the sample.)
7. Centrifuge the resulting supernatant for 20 min at 47,000*g* at 4°C to collect the PSII-enriched sample.
8. Wash the sample (*see* **Note 2**) twice in standard buffer using four centrifuge tubes in a Sorval SS-34 rotor at 4°C to remove the excess detergent.
9. Resuspend the combined pellet in a small volume of standard buffer to a sample concentration of roughly 2–4 mg Chl/mL and stored until use (*see* **Note 3**).

3.1.2. Chlorophyll Determination

1. Determine the chlorophyll concentration of a sample according to the procedure of Porra et al. ***(5)***.

2. Extract the chlorophyll pigments from a 10–20 µL aliquot of the sample in 5 mL of 80% acetone solution.
3. Vortex the solution for one minute and then centrifuge in a clinical centrifuge at 1000*g* for one minute to remove any undissolved material. (The supernatant should be pale green while the precipitated protein material will appear light brown.)
4. Measure the absorbance of the clarified supernatant in a spectophotometer at 646.6 nm, 663.6 nm, and 750 nm. (Ideally, sample dilutions should be used to give absorbances in the range of 0.3 to 1.2 O.D. units for accurate determinations; however, higher absorbances may be tolerated depending on the quality of the spectrophotometer.)
5. The total chlorophyll ($a + b$) is calculated as:

$$\text{Chl } [\mu\text{g/mL}] = [17.76\,(\text{Abs}_{646.6} - \text{Abs}_{750}) + 7.34(\text{Abs}_{663.6} - \text{Abs}_{750})] \times \text{DF}$$

where DF is the dilution factor (i.e., 250–500).

3.2. Extraction Procedures

3.2.1 Complete Extraction of Mn

The following procedure is used to completely extract the functional Mn from PSII. It is basically the same procedure reported earlier by Hunziker et al. *(6)*, but with some slight modifications (G. C. Dismukes, personal communication, January 2003).

1. Thaw approx 5 mg Chl of the frozen PSII sample and wash once in 50 mL of standard buffer. Resuspend the pellet in a small volume of low MES standard buffer and the Chl concentration is determined.
2. Centrifuge an aliquot containing 4 mg Chl of the sample at 34,500*g*. Resuspend the pellet in 16 mL of wash buffer 1 to extract the Mn (in which the final sample concentration will be 0.25 mg Chl/mL) and gently stir at room temperature under room light (5–10 µE) for 10 min.
3. The sample is then diluted 1 / 1 with 50 m*M* MES buffer, pH 6.5, to stop the reaction and then centrifuge at 34,500*g*.
4. Wash the pellet sample once in standard buffer to which 1 m*M* EDTA has been added (*see* **Note 4**).
5. Subsequently, the pellet sample is washed in chelex-treated standard buffer (*see* **Note 5**) and resuspend in the same buffer to about 2–4 mg Chl/mL for analysis or storage (*see* **Note 3**).

3.2.2 Partial Extraction of Mn

The procedure for the partial extraction of the functional Mn is similar to the ones reported by Kuwabara and Murata *(7)* and Tamura and Cheniae *(8)*.

1. Thaw approx 5 mg Chl of the frozen PSII sample and wash once in 50 mL of standard buffer. Resuspend the pellet in a small volume of standard buffer and the Chl concentration is determined.

2. An aliquot containing 4 mg Chl of the sample is centrifuge at 34,500*g*. Resuspend the pellet in 16 mL of wash buffer 2 to partially extract Mn (in which the final sample concentration will be 0.25 mg Chl/mL) and gently stir on ice in the dark for 10 min.
3. Wash the pellet sample twice in chelex-treated standard buffer (*see* **Note 5**) and resuspend in the same buffer to about 2–4 mg Chl/mL for analysis or storage (*see* **Note 3**).

3.2.3 Specific Extraction of Ca

The procedure for the specific extraction of the functional Ca is the same as the one reported by Latimer et al. ***(9)***.

1. Thaw approx 10 mg Chl of the frozen PSII sample and wash once in 50 mL of standard buffer. Resuspend the pellet in a small volume of low MES standard buffer and determine the Chl concentration and adjust to 4 mg Chl/mL.
2. Dilute a 2 mL aliquot containing 8 mg Chl of the sample with 2 mL of wash buffer 3 to specifically extract the Ca and stir in a 0°C ice bath (*see* **Note 6**) in the dark for 5 min.
3. Dilute the sample with 4 mL of high MES standard buffer to which 100 μ*M* EDTA has been added and centrifuge at 34,500*g*.
4. Wash the pelleted sample twice in Chelex-treated standard buffer (*see* **Note 5**) and resuspend in the same buffer to about 2–4 mg Chl/mL for analysis or storage (*see* **Note 3**).

3.3. Analysis Procedures

3.3.1. O_2 Evolution Activity

1. Measure the O_2 evolution activity using a standard Clark O_2 electrode set-up (e.g., Hansatech) ***(10)***.
2. Add an aliquot of the PSII sample containing 10–20 μg Chl to a water-jacketed assay vessel that contains 1 mL of the assay buffer, which includes $K_3Fe(CN)_6$ and PPBQ as electron acceptors.
3. Connect the water-jacketed assay vessel to a 25°C circulating water bath and allow the sample solution to temperature equilibrate in the dark for a few minutes.
4. Continuously illuminate the sample utilizing a strong actinic white light (e.g., a 200 W projector lamp) (*see* **Note 7**) for at least 1 min and record the amount of O_2 evolved by the sample as a function of time.
5. Calibrate the O_2 signal from the sample in terms of μmoles O_2 by using the signal difference between air-saturated water at 25°C that contains 0.253 μmoles O_2 mL^{-1} and after bubbling the air-saturated water with nitrogen gas to remove O_2. Calculate the initial rate (linear part) of O_2 signal produced by the sample in the light as μmoles O_2 (mg Chl)$^{-1}$ h^{-1} (*see* **Note 9**).

As shown in **Table 1**, the untreated PSII preparation has an O_2 evolution activity of about 700 μmoles O_2 (mg Chl)$^{-1}$ h^{-1}. Depending on the source of

Table 1
O_2 Evolution Activity of Variously Extracted Photosystem II Preparations

Sample	Activity [µmol O_2 (mg Chl)$^{-1}$ h^{-1}]
Control	722 ± 10
Complete Mn Extracted	0
Partial Mn Extracted	0
Photoactivated	259
Specific Ca Extracted	61
Ca Reconstituted	290

the plant material and the sample preparation, control rates can vary between 600 and 900 µmoles O_2 (mg Chl)$^{-1}$ h^{-1}. Upon complete extraction of the Mn, the O_2 evolution drops to zero, or the sample may show a slight O_2 uptake. Such samples can be reactivated to varying extents by adding back Mn and Ca to the sample under turnover conditions using light flashes *(11)*. Upon partial removal of the Mn, the activity drops to 0–10% of the control. As indicated in **Table 1**, for the samples reported here, no residual O_2 evolution is observed after partial Mn extraction. As is also shown in **Table 1** O_2 evolution can then be reactivated to 37% of the control by incubating in standard buffer to which 1 m*M* $MnCl_2$, 20 m*M* $CaCl_2$, 10 µ*M* DCPIP (2,6-dichlorophenolindophenol) has been added in the presence of weak light (30 µE) ***(11)***. Similarly, upon the specific removal of Ca the O_2 evolution activity is also mostly lost and drops to 7% of the control for the sample shown in **Table 1**. In this case, the activity can be restored to 60% of the control by simply incubating the sample in the standard buffer to which 20 m*M* $CaCl_2$ h as been added for 1–2 h on ice in the dark *(12)*.

3.3.2. Protein Composition

The protein composition of PSII samples is determined by standard SDS-PAGE (e.g., using a Biorad system) and the basic procedure of Lemmli *(13)*. The major protein subunits in PSII are visualized in 10–17.5% gradient polyacrylamide-resolving gel to which 6 *M* urea has been added.

1. PSII proteins are first solubilized by incubating two parts of the sample containing 1 mg Chl mL^{-1} with one part of 3 × 2% SDS solubilization buffer for 1 h at room temperature.

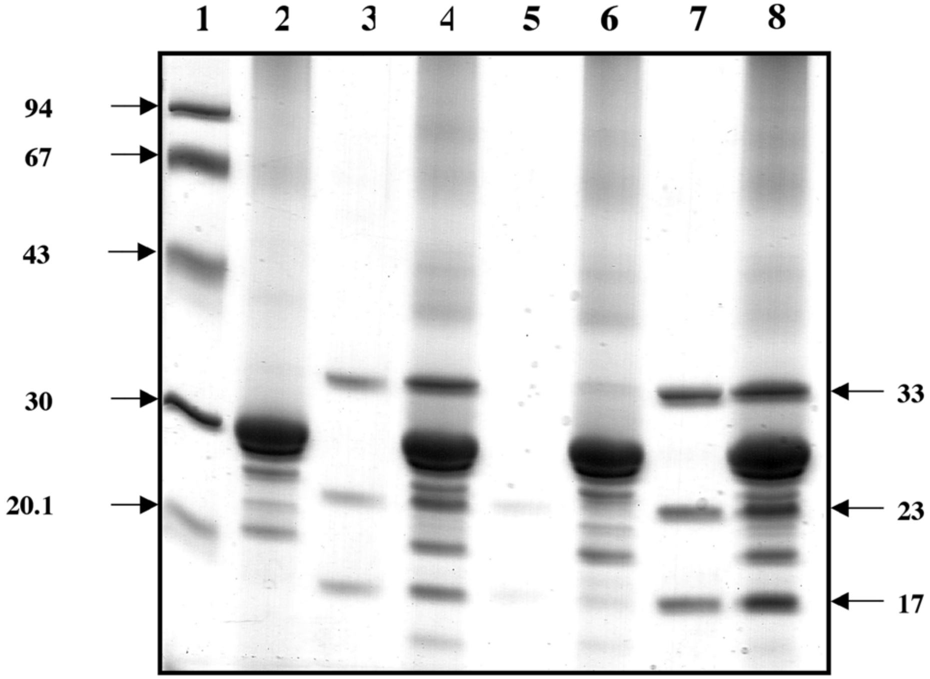

Fig. 1. Protein composition for variously extracted photosystem II preparations. Lane 1, Standards; Lane 2, Complete Mn Extracted—Pellet; Lane 3, Complete Mn Extracted—Supernatant; Lane 4, Partial Mn Extracted—Pellet; Lane 5, Partial Mn Extracted—Supernatant; Lane 6, Specific Ca Extracted—Pellet; Lane 7, Specific Ca Extracted—Supernatant; Lane 8, Control.

2. A 20 μL aliquot of the solubilized sample is then applied to a well in a 6% polyacrylamide stacking gel and electrophoresed for about 8 h at 2–3 W constant power or about 16 h at 1 W constant power.
3. The resolving gel is stained with Comassie brilliant blue and destained in the standard glacial acetic acid/methanol solution.

Upon complete extraction of the functional Mn, three proteins of apparent molecular masses of 33, 23, and 17 kDa are released. This is evidenced in **Fig. 1** by comparison of the extracted pellet in lane 2 with the supernatant in lane 3. These three proteins are known to form an extrinsic cap on the lumenal surface of the PSII complex, which shields the Mn_4Ca core from the aqueous solvent phase ***(14)***. The 33 kDa protein is often called the manganese stabilizing protein (MSP) and is essential for keeping the functional Mn intact, whereas the

23 kDa protein is believed to enhance the binding affinity for Ca. In this procedure, the metal ions are released from PSII as the Mn_4Ca core dissociates as a result of exposure to the aqueous solvent.

Upon the partial extraction of the functional Mn using hydroxylamine treatment, there is minimal loss of the extrinsic proteins, so that the structure of PSII remains mostly intact. This is shown in **Fig. 1** by comparison of the extracted pellet in lane 4 with the supernatant in lane 5. In this procedure, the hydroxylamine chemically reduces the functional Mn to Mn(II) which has a much weaker binding affinity to the OEC and is readily released from the catalytic site.

Finally, upon the specific extraction of the functional Ca, the 33, 23, and 17 kDa extrinsic proteins are also released. This is shown in **Fig. 1** by comparison of the extracted pellet in lane 6 with the supernatant in lane 7. In this procedure, the low pH used during the treatment reversibly dissociates the three extrinsic proteins, which allows the Ca to be released as the citrate bridges the Ca binding site to stabilize the Mn_4 cluster. Upon neutralizing the pH, the extrinsic proteins rebind to PSII ***(15)*** and are stabilized by the added Ca; hence, a significant part of the activity is restored. As shown by the untreated control sample in lane 8 in **Fig. 1**, the other proteins characteristic of PSII are little affected by these extraction procedures.

3.3.3 Metal Content

To determine the Mn and Ca content, samples are acid digested and the Mn and Ca are measured by atomic absorption methods.

1. To remove extraneous, nonspecifically bound metal ions, mix 2 g of Chelex 100 with an aliquot of sample containing 2 mg Chl in 4 mL of 25 m*M* MES/NaOH buffer, pH 6.5, and 15 m*M* NaCl.
2. Stir the solution gently for 1 h at 4°C in the dark.
3. Filter through a nylon cloth (20 µ mesh), that has been washed in chelexed, deionized water, to remove the Chelex 100 beads.
4. Centifuge the sample at 47,000*g* in a Sorvall SS-34 rotor and resuspend the pellet in a small volume of chelexed standard buffer (*see* **Note 8**).
5. Determine the sample concentration and treat the pellet of a 1 mg Chl aliquot with 2.5 mL concentrated nitric acid and 0.5 mL perchloric acid.
6. Using a glass rod, disperse the sample, then incubate for 2 h in a 50°C water bath.
7. Bring the digested sample to room temperature in a refrigerator and sonicate to break up any undissolved material.
8. Bring the volume up to 10 mL with chlelexed, deionized water.
9. Analyze the sample by atomic absorption methods to determine the Mn and Ca content.

As shown in **Table 2**, the control samples reported here contain 4.5 Mn per 200 Chl (*see* **Note 9**) as expected. Upon complete extraction of the functional

Table 2
Metal Contents of Variously Extracted Photosystem II Preparations

		Ca/200 Chl	
		This (acid digest and chelex treated) work	Reference 17 (chelex treated)
Control	4.5	22.0	2.1
Complete Mn Extracted	0.7	—	—
Partial Mn Extracted	1.9	—	—
Specific Ca Extracted	—	11.5	1.1

Mn this value drops to 0.7 Mn per 200 Chl. Upon partial removal of the functional Mn, the Mn content drops to 1.9 Mn per 200 Chl.

The Ca content is difficult to evaluate as there are many sources of possible contamination that can interfere with the quantification; from the glassware used, to residual metals present in the acids needed to digest the sample. For the control sample reported here carried through the acid digestion procedure, about 22 Ca per 200 Chl were found. Upon the removal of the functional Ca, the value drops to one-half or about 11 Ca per 200 Chl. It is expected that one-half of the bound Ca per PSII is removed by this procedure. By using the approach of Kashino et al. ***(16)***, Shen et al. ***(17)*** reported 2.1 Ca per 200 Chl for untreated samples and 1.1 Ca per 200 Chl after the low pH/citrate treatment. However, in this approach, the samples are not acid digested but are only chelexed treated and the Ca released into the supernatant is determined by atomic absorption.

4. Notes

1. The pellets from thylakoid and PSII-enriched samples should be gently dispersed using a camel's hair brush. An appropriate size brush should be chosen to fit the size of the pellet. Dispersion of the sample by a vortex mixer should be avoided as severe mechanical motions can inactivate the sample.
2. Throughout the various protocols, "washing" implies resuspension of the sample in the appropriate buffer at 0.25 mg Chl mL^{-1} followed by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 10 min at 4°C in the dark, unless otherwise stated.
3. Samples can be quick-frozen as small beads by dropping the sample from a Pasteur pipet into liquid nitrogen. The frozen beads can be collected using a metal strainer and quickly transferred to a –80°C ultra-cold freezer.
4. A stock solution of 100 m*M* EDTA should be prepared by dissolving 3.76 g of the disodium salt in 100 mL of deionized water at pH 8.0.

5. For chelexed treated solutions, 10 g of Chelex 100 is added to 1 L and allowed to incubate for at least 1 d. Chelex 100 is prepared by washing the resin in a column with four bed volumes of deionized water, four bed volumes of 0.5 *M* sodium acetate at pH 6.5 and four bed volumes of deionized water.
6. To achieve a 0°C ice bath, 5–10 g of salt is added to 1 L of crushed ice. As the sample solution incubates in the 0°C ice bath, make sure that it doesn't freeze by maintaining a gentle stirring.
7. When making light-induced O_2 activity measurements, make sure that the actinic light is saturating by focusing the light onto the sample chamber through a lens or fiber optic. It is best to measure the light saturation curve for the measurements using a set of calibrated neutral density filters.
8. Glassware should be acid washed and thoroughly rinsed in deionized water to remove contaminating metal ions. This is particularly important for the metal assays. To acid wash, the glassware is soaked for at least a day in 5% nitric acid.
9. To determine the metal content in terms of 200 Chl (the estimated photosynthetic unit size for a typical PSII preparation), the mole fraction of the metal is determined per mole Chl of the starting sample multiplied by 200. Use the atomic weights of 54.94 and 40.08 for Mn and Ca, respectively, and an average molecular weight of 900 for Chl.

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21

Assay of Photoinhibition of Photosystem II and Protease Activity

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Summary

Under light-stress conditions, the photosystem (PS)II reaction center D1 protein is photodamaged. The damage to the D1 protein is induced by singlet oxygen molecules and endogenous free radicals generated by the photochemical reactions of PSII. To maintain PSII activity, the oxidatively damaged D1 protein is replaced by a newly synthesized protein. Thus, degradation and removal of the photodamaged D1 protein in PSII are essential steps for maintaining the viability of PSII. In the present chapter, we describe the method to induce photoinhibition of PSII both in vitro and in vivo, and also the method to assay the processes closely related to the photoinhibition, including degradation of the damaged D1 protein and its crosslinking with the neighboring polypeptides. The method to analyze the protease activity in the stroma that recognizes and digests the crosslinked products of the D1 protein generated by the light stress is also described.

Key Words: D1 protein; light stress; photosystem (PS)II; photoinhibition; protease; protein crosslinking; protein degradation; protein purification reactive oxygen species; Western blot analysis.

1. Introduction

Illumination of active oxygen-evolving photosystem (PS)II samples with excessive light results in the full reduction of the plastoquinone electron acceptors, Q_A and Q_B, of PSII, which induces release of the fully reduced plastoquinones from their binding sites. This release stimulates charge recombination between the reduced primary electron acceptor pheophytin and the oxidized primary electron donor $P680^+$. This charge recombination process generates a triplet state of P680, which then reacts with oxygen to form singlet oxygen molecules under aerobic conditions. The primary cause of photodamage to the

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D1 protein under excessive illumination of healthy leaves, thylakoids, and PSII samples is, thus, the singlet oxygen produced in the overexcited PSII reaction center, and this is called the acceptor-side photoinhibition of PSII ***(1,2)***. The events occurring in the acceptor-side photoinhibition of PSII are the primary cleavage of the D1 protein to an N-terminal 23-kDa and a C-terminal 10-kDa fragments, the secondary degradation of these fragments, and extensive crosslinking of the photodamaged D1 protein with the surrounding polypeptides in PSII ***(3)***. In the pioneering work of Greenberg et al. ***(4)***, which showed a 23.5 kDa breakdown product of the D1 protein with *Spirodela oligorrhiza*, the primary cleavage of the D1 protein was shown to take place at the stroma-exposed DE-loop of the D1 protein. The degradation process of the D1 protein is not clear yet, although several candidates are proposed for the proteolytic enzymes ***(5)***. As to the crosslinking of the photodamaged D1 protein, several crosslinked products were identified, namely D1/D2, D1/the α-subunit of cytochrome b_{559}, and D1/CP43 have been identified ***(6)***, but the specific crosslinking sites remain to be identified. Judging from the data that the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibits the protein crosslinking, we assume that the Q_B binding site of the D1 protein may be involved in the event. Because steps related to the acceptor-side mechanism of photoinhibition of PSII are dependent on oxygen, these experiments typically are carried out under aerobic conditions.

From in vitro studies, it has been shown that oxygen evolving system of PSII is impaired under some stress conditions such as high or low temperature conditions, and Ca^{2+}- or Cl^--depleted conditions. When PSII is illuminated under these stress conditions, endogenous radicals are generated at the donor side of PSII that originate from oxidation of the primary electron donor, $P680^+$, and the secondary electron donor, Tyr Z^+, of PSII. These radicals induce significant damage to the D1 protein at the lumen-exposed portion, and this is called the donor-side photoinhibition of PSII ***(1,2)***. As the primary cause of the photodamage in the donor-side photoinhibition of PSII is the generation of cationic radicals, the process itself should not be dependent on oxygen, and therefore the stress-related events are observed even under anaerobic conditions ***(3)***. It was suggested that cleavage of the D1 protein takes place at the lumen-exposed AB-loop of the D1 protein, and the 10-kDa N-terminal and a 23-kDa C-terminal fragments are produced ***(2)***. However, as far as the donor-side photoinhibition is induced under anaerobic conditions, the crosslinked products of the D1 protein and the antenna chlorophyll-binding protein CP43 are the major products ***(7)***.

The photodamaged D1 protein is degraded by the action of proteases in the chloroplasts. So far, only the proteases involved in the degradation of the damaged D1 protein in the acceptor-side photoinhibition of PSII are known, and no protease is reported that recognizes the crosslinked or cleavage products of the D1 protein generated by the donor-side photoinhibition. It was proposed that the primary cleavage of the D1 protein into the 23 kDa and 10 kDa fragments in the acceptor side photoinhibition is carried out by the DegP2 protease, which is loosely bound to the stroma-exposed surface of the nonstacked region of the thylakoids ***(8)***. The 23 kDa fragment is suggested to be digested by the FtsH protease bound to the thylakoids ***(9)***. These successive proteolytic steps may be involved in a specific pathway for proteolyzing photodamaged D1 protein. A recently proposed alternative and novel pathway of D1 degradation involves rather significant cross-linking of the photodamaged D1 protein and the surrounding polypeptides in PSII, which is followed by digestion of the crosslinked products by stromal protease(s) ***(3,6,10)***.

The events of photodamage and protein processing in both the donor-side and acceptor-side photoinhibition mechanisms are analyzed by sodium dodecyl sulfate (SDS)/urea-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. The use of specific antibodies against the D1 protein and other PSII proteins, and fluorography with enhanced chemiluminescence (ECL), is employed routinely as an alternative to previous radioactivity-based assays. In this chapter, we describe a method to induce photoinhibition of PSII, both in vitro and in vivo, and to detect the crosslinked, cleavage, and degradation products of the photodamaged D1 protein. An assay for the protease activity specifically degrading the crosslinked products of the D1 protein also is described.

2. Materials

2.1. Preparation of PSII-Enriched Membranes

Spinach or pea leaves. The details of the materials and methods for preparation of these samples are shown elsewhere in the same volume ***(3)*** (*see* **Note 1**).

2.2. Preparation of Leaf Discs

1. Spinach or pea leaves.
2. 3 m*M* Chloramphenicol or 2 m*M* lincomycin.
3. A cork borer.

2.3. Preparation of Intact Chloroplasts

1. Spinach or pea leaves.
2. 5X Grinding medium (GM) stock solution: 1.65 *M* sorbitol, 5 m*M* $MgCl_2$, 5 m*M* $MnCl_2$, 10 m*M* EDTA, 0.25 *M* Hepes-KOH (pH 7.5).
3. 1X GM: 0.33 *M* sorbitol, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 2 m*M* EDTA, 50 m*M* Hepes-KOH, 0.25% (w/v) BSA, 5 m*M* Na-ascorbate.
4. ST buffer: 0.33 *M* sorbitol, 50 m*M* Tricine-KOH (pH 8.0).
5. PBF-Percoll: 0.56 g polyethylene glycol no. 4000, 0.185 g BSA, 0.185 g Ficoll, 18.5 mL Percoll.
6. 0.5 *M* Na-ascorbate: 0.5 *M* Na-ascorbate, 50 m*M* Hepes-KOH (pH 7.5).

7. Percoll gradient (20%): 1.5 mL 5X GM, 1.5 mL PBF-Percoll, 1.5 mg glutathione (reduced form), 75 µL 0.5 *M* Na-ascorbate, 4.5 mL distilled water (total volume, 7.5 mL).
8. Percoll gradient (50%): 3.6 mL 5X GM, 9.0 mL PBF-Percoll, 3.6 mg glutathione, 180 µL 0.5 *M* Na-ascorbate, distilled water (total volume, 18 mL).
9. Percoll gradient (80%): 2.0 mL 5X GM, 8.0 mL PBF-Percoll, 2 mg glutathione, 100 µL 0.5 *M* Na-ascorbate (total volume, 10 mL).
10. A blender.
11. A centrifuge and a swinging bucket rotor.

2.4. Preparation of the Stromal Fraction and Intact Thylakoids

1. Lysis buffer (LB): 5 m*M* $MgCl_2$, 10 m*M* Mes-NaOH (pH 6.5).
2. TC buffer: 50 m*M* sorbitol, 50 m*M* Tricine-KOH (pH 8.0), 15 m*M* NaCl, 5 m*M* $MgCl_2$.
3. An ultracentrifuge.

2.5. SDS/Urea-PAGE

1. Acrylamide stock solution: 50% (w/v) acrylamide, 13.3% (w/v) bis-acrylamide (total volume, 500 mL).
2. Resolving gel solution: 12.5% acrylamide (5 mL acrylamide stock solution), 6 *M* urea, 0.1% (w/v) SDS, 0.6 *M* Tris-HCl (pH 8.8) (total volume, 20 mL).
3. Stacking gel solution: 4.5% acrylamide (0.9 mL acrylamide stock sokution), 6 *M* urea, 0.1% (w/v) SDS, 0.125 *M* Tris-HCl (pH 6.8) (total volume, 10 mL).
4. Electrophoresis buffer: 25 m*M* Tris-HCl, 0.192 *M* glycine, 0.1% (w/v) SDS (pH adjustment not necessary) (total volume, 1/l).
5. Lysis buffer: 6 *M* urea, 5% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 5 m*M* EDTA, 5% (w/v) sucrose, 0.125 *M* Tris-HCl (pH 6.8) (total volume, 30 mL).
6. Ammonium persulfate solution: 10% ammonium persulfate.
7. TEMED (*N*,*N*,*N'*,*N'*-tetramethylene-ethylenediamine) solution.
8. Staining solution: 0.05% Coomassie brilliant blue R-250, 50% methanol, 7% acetic acid.
9. Destaining solution: 20% methanol, 7% acetic acid.
10. Molecular-weight marker proteins (a low-molecular-weight electrophoresis calibration kit available from Pharmacia, Sweden).
11. A vertical electrophoresis apparatus and a power supply.

2.6. Western Blot Analysis

1. Transfer buffer: 0.192 *M* glycine, 25 m*M* Tris-HCl (pH 7.4), 10% (v/v) methanol.
2. TBS: 0.15 *M* NaCl, 20 m*M* Tris-HCl (pH 7.4).
3. TTBS: 0.15 *M* NaCl, 0.05% (v/v) Tween-20, 20 m*M* Tris-HCl (pH 7.4).
4. Blocking buffer: 5% (v/v) skim milk, 0.02% (v/v) NaN_3, 0.15 *M* NaCl, 0.05% (v/v) Tween-20, 20 m*M* Tris-HCl (pH 7.4).
5. The first antibody solution: the first antibody (raised in a rabbit) diluted according to its titer, 1% (w/v) BSA, 0.01% (w/v) NaN_3, 0.15 *M* NaCl, 0.05% (v/v) Tween-20, 20 m*M* Tris-HCl (pH 7.4).

6. The second antibody solution: 1% (w/v) skim milk, 0.01% (w/v) the second antibody (horse radish peroxidase-coupled anti-rabbit goat antibody purchased from BioRad), 0.15 *M* NaCl, 0.05% (v/v) Tween-20, 20 m*M* Tris-HCl (pH 7.4).
7. Probe-removing solution: 2% (w/v) SDS, 100 m*M* β-mercaptoethanol, 62.5 m*M* Tris-HCl (pH 6.7).
8. PVDF membranes (Millipore).
9. An electroblotting apparatus.
10. A power supply.

2.7. Activity Gel (Gelatin/SDS-PAGE)

1. Gelatin/SDS-PAGE: 0.2% (w/v) gelatin in the resolving gel of the SDS-PAGE (*see* **Note 2**).
2. The stromal fraction of intact chloroplasts.
3. Renaturing buffer: 50 m*M* Tris-HCl (pH 8.0), 5 m*M* $MgCl_2$, 1% (w/v) Triton-X100.
4. Incubation buffer: 50 m*M* Tris-HCl (pH 8.0), 5 m*M* $MgCl_2$.
5. Staining solution (*see* **Subheading 2.5.**).
6. Destaining solution (*see* **Subheading 2.5.**).
7. Lysis buffer (LB) (*see* **Subheading 2.4.**).
8. An electrophoresis apparatus.
9. A knife or razor blade.
10. A glass homogenizer (a Teflon pestle and a glass container).

3. Methods

3.1. Preparation of Leaf Discs

1. Prior to cutting-out leaf discs, immerse spinach leaf petioles in a solution containing chloramphenicol (3 m*M*) or lincomycin (2 m*M*) in the dark for 3 h to inhibit the synthesis of the D1 protein.
2. Cut the fresh leaves with a cork borer at a diameter of 1 cm.

3.2. Preparation of Intact Chloroplasts

1. Rinse spinach leaves with tap water and cool them in ice.
2. Cut the leaves in squares (2 cm × 2 cm) with a knife.
3. Homogenize the leaves with a pre-cooled blender. Add 500 mL GM per 100 g spinach leaves.
4. Filter the homogenate with four layers of cheese cloth.
5. Centrifuge the filtrate at 3000*g* for 90 min.
6. Suspend the precipitates with a small volume of GM.
7. Load the suspension on a Percoll gradient (20–50–80%) and centrifuge in a swinging bucket rotor at 5500*g* for 10 min.
8. The intact chloroplasts form a green band at the interface between the 50 and 80% Percoll. Collect the band with a pipet.
9. Dilute the intact chloroplast fraction with 5 volumes of ST.
10. Centrifuge at 1000*g* for 90 s and obtain the intact chloroplasts as a precipitate.

3.3. Preparation of the Stromal Fraction and Intact Thylakoids

1. Suspend the isolated intact chloroplasts in LB at a chlorophyll concentration of 2 mg/mL, and incubate for 5 min on ice.
2. Centrifuge the suspension at 40,000*g* for 20 min; the supernatant is the stromal fraction.
3. Wash the precipitate with LB and suspend in TC at a chlorophyll concentration of 0.5 mg/mL. This suspension is used as intact thylakoids.

3.4. Photoinhibitory Illumination

1. Induce photoinhibition of PSII by visible light by a conventional light source such as a slide projector with a 1000 W tungsten lamp. Set heat cutoff filters between the lamp and the samples.
2. Samples are placed in transparent plastic sample tubes (1 mL volume) for incubation at a specific temperature, which are then placed in a reaction vessel connected to a temperature-controlled circulating water bath. We constructed the reaction vessel from transparent acrylic plates.
3. Illuminate the sample tubes by light from the side or the top. Float the leaf discs on the surface of the water in the reaction vessel and illuminate from the top. To avoid the diffusion of CO_2 through stomata becoming a rate-limiting factor for overall photosynthesis, place the leaf discs upside-down on the water surface and illuminate them from the bottom. For this purpose, the transparent reaction vessel is put on an overhead projector. The light intensity is measured with a quantum sensor QSPAR (Hansatech, UK). The leaf discs are then homogenized and subjected to the assay of photoinhibition (*see* **Note 3**).

3.5. SDS/Urea-PAGE

The method is based on Laemmli *(11)*. A 12.5% acrylamide gel is used here but a gradient gel (for instance from 10 to 20%) can also be used. To improve resolution, 6 *M* urea is included both in the stacking and resolving gels, and 0.6 *M* Tris-HCl *(12)* is used in the resolving gel. Samples are solubilized in LB also containing 6 *M* urea. For the electrophoresis conditions, *see* **Note 4**.

3.6. Assay of the Crosslinked Products of the D1 Protein Generated by the Acceptor-Side Photoinhibition of PSII With Western Blot Analysis and Fluorography

1. Separate the proteins of the samples by SDS/urea-PAGE.
2. The proteins in an SDS/urea-PAGE gel are transferred to a PVDF membrane. The power supply is set to 200 mA (constant current) and run for 1 h at 4°C.
3. We use a wet-type blotting apparatus because proteins are evenly transferred to the PVDF membranes.
4. Incubate the PVDF membrane in TBS for 5 min with shaking. A small plastic container can be used for the incubation.
5. Incubate the membrane in blocking solution for 1 h with shaking.

6. Rinse the membrane twice with TTBS.
7. Incubate the membrane in a solution containing the first antibody for 1 h with shaking.
8. Rinse the membrane twice with TTBS, then incubate once with TTBS for 15 min and twice for 5 min. Incubate the membrane in the solution containing the second antibody for 1 h.
9. Rinse the membrane with TTBS.
10. Transfer the membrane to another plastic container and add the ECL detection solution. The ECL detection solution is prepared by mixing the equal volumes of two reagents (denoted solutions 1 and 2). Use 4 mL of the solution for one 10 cm × 10 cm membrane. Incubate for 1 min.
11. Place the PVDF membrane in a plastic sack and seal it with a sealer.
12. Under safe light in a dark room, lay the sack with the membrane in an X-ray film cassette and place a piece of X-ray film over the sack.
13. After exposure of the film to the PVDF membrane for an appropriate period, develop the film with an X-ray film developer (**Fig. 1**).
14. To reuse the membrane, remove the antibodies; incubate the membrane in 30–50 mL of the probe-removing solution for 1 h at 50°C with shaking in a tightly covered plastic container (*see* **Note 5**).
15. Transfer the membrane to another container and rinse the membrane several times with TTBS.
16. Incubate the PVDF membrane with TTBS for several minutes with shaking. Repeat this step until the β-mercaptoethanol odor is no longer present.
17. The membrane is ready for probing with another antibody, beginning with **step 4** in **Subheading 3.6.** If the membrane is not to be used immediately, store it in blocking solution and in a refrigerator.

3.7. Assay of Protease Activity That Recognizes the Crosslinked Product of the D1 Protein

1. Illuminate the samples with strong light.
2. Add the stromal fraction at room temperature and incubate for a given time.
3. Stop the reaction by adding SDS/urea-PAGE lysis buffer.
4. Analyze the proteins by SDS/urea-PAGE and Western blot analysis with the antibody against the D1 protein.
5. The change in the amounts of the D1 protein crosslinked products is detected by fluorography in the presence and absence of the stromal fraction (**Fig. 1**).

3.8. Electrophoretic Separation of an SDS-Resistant Stromal Protease(S) by the Gelatin Activity Gel Method

1. Prepare a gelatin/SDS/urea-polyacrylamide gel.
2. Subject the stromal fraction to gelatin/SDS/urea-PAGE at 4°C.
3. Remove the SDS from the gel by incubating it in renaturation buffer at 25°C for 30 min; then transfer to incubation buffer at 37°C for 16 h (*see* **Note 6**).
4. Stain the gel with Coomassie brilliant blue. Proteases are detected as transparent bands in the gel where the gelatin has been digested by protease activity (**Fig. 2**).

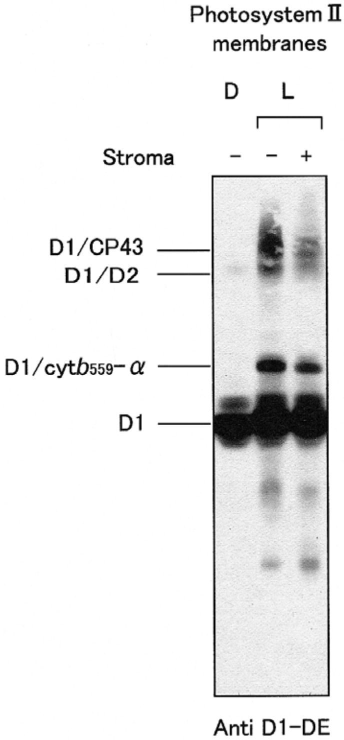
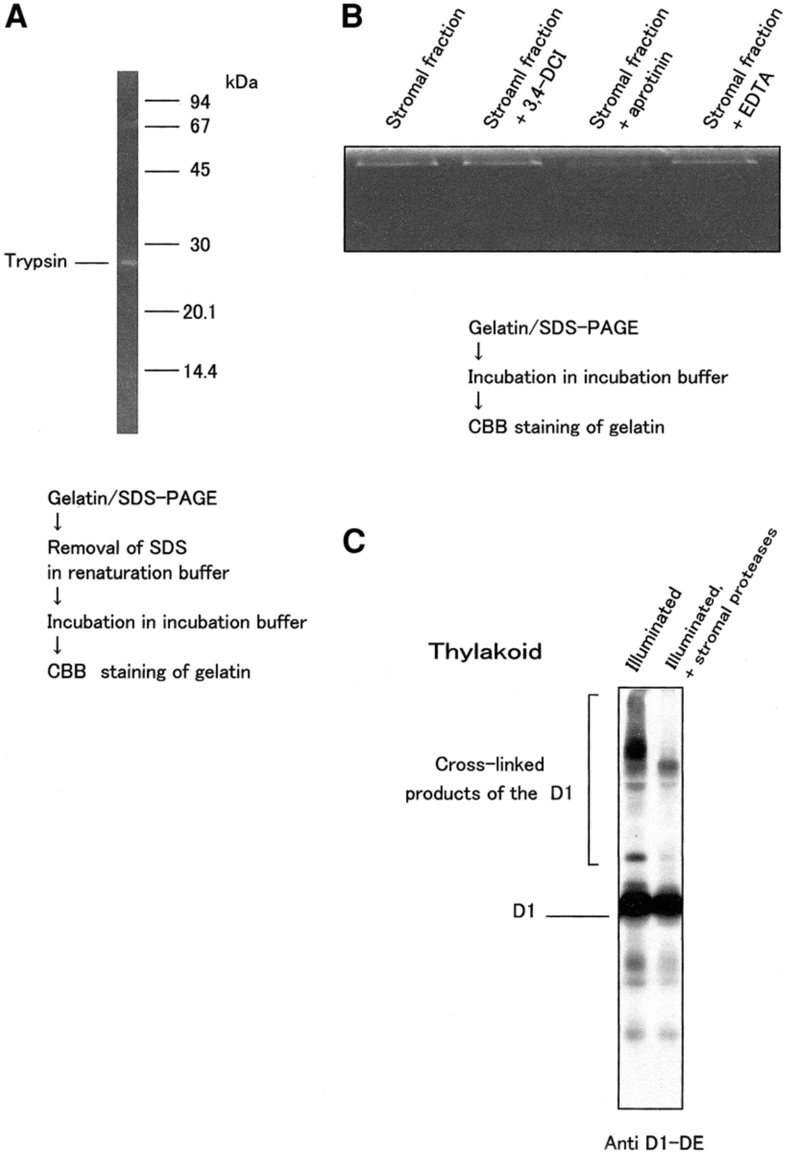


Fig. 1. Profile of the crosslinked products of the D1 protein generated by the illumination of spinach photosystem II membranes. The photosystem II membranes were illuminated with white light (2000 µE m^{-2} s^{-1}) for 60 min at 25°C. Where indicated, the samples were further incubated with the stromal fraction for 60 min at 25°C. The crosslinked products of the D1 proteins were detected by SDS/urea-PAGE and Western blot analysis with specific antibody against the DE-loop of the D1 protein. D and L denote dark and light, respectively. The mobilities of the native D1 protein, the 41-kDa crosslinked product of the D1 protein and the α-subunit of cyt b_{559}, the D1/D2 heterodimer and the D1/CP43 crosslinked products are indicated at the left side of the gel.

Fig. 2. (*Opposite page*) Separation of an SDS-resistant stromal protease(s) in the stroma by gelatin/SDS-PAGE and examination of the effects of the protease(s) on the crosslinked products of the D1 protein. **(A)** As a control experiment, trypsin was separated by a standard gelatin/SDS-PAGE. Trypsin was detected as a white band in a blue background by Coomassie staining. The relative molecular masses of marker proteins are shown at the right side of the gel. **(B)** The SDS-resistant protease(s) in the stroma was detected as a white band at the top of the separation gel, as was reported previously by Sokolenko et al. ***(13)***. The protease activity was inhibited by the addition of aprotinin, a serine-type protease inhibitor, while 3,4-dichloroisocoumarin (DCI), another serine-type protease inhibitor, and EDTA, an inhibitor of metalloproteases, had no effect



on the protease activity. The concentrations of 3,4-DCI, aprotinin and EDTA were 20 μ*M*, 5 μ*M* and 1.4 m*M*, respectively. **(C)** The effects of the stromal protease(s) on the crosslinked products of the D1 protein were examined by SDS/urea-PAGE and Western blot analysis with a specific antibody againt the DE-loop of the D1 protein. Spinach thylakoids were illuminated with white light (2000 μE m^{-2} s^{-1}) for 90 min at 25°C. Where indicated, the illuminated thylakoids were further incubated with the isolated protease(s) for 60 min at 25°C. The protease(s) significantly digested the crosslinked products of the D1 protein.

5. To extract the proteases from the gel, the gel is not stained with Coomassie, and is homogenized with a glass homogenizer. Proteases are extracted from the homogenate.
6. The extracted protease activity can be monitored by several methods, including a sensitive fluorescence assay (e.g., EnzChek Protease Assay Kits are available from Molecular Probes Inc.).

4. Notes

1. When preparing the membrane samples, spinach or pea leaves are usually placed in a dark cold room overnight to deplete starch in the chloroplasts. It should be noted that all the sample preparation steps are carried out at 4°C in the dark under green safe light, because unnecessary exposure of the samples to light may affect the results of photoinhibition studies. After preparation, the samples are immediately frozen in liquid nitrogen and stored at –80°C. During storage, the samples are kept in black sample tubes (or in tubes or bottles shielded from light by wrapping with aluminum foil).
2. For the conventional SDS-PAGE, urea is omitted from the gels and lysis buffer shown in **Subheading 2.5.**
3. For the assay of degradation and crosslinking of the D1 protein in the illuminated leaf discs, the leaf discs are homogenized in 5 mL of a solution containing 0.1 *M* sucrose, 0.2 *M* NaCl and 50 m*M* KH_2PO_4 (pH 6.9). The homogenate is quickly filtered through two layers of tissue and centrifuged at 10000*g* for 5 min. The pellet is suspended in a buffer solution containing 0.1 *M* sorbitol, 15 m*M* NaCl, 5 m*M* $MgCl_2$ and 50 m*M* Tricine-KOH (pH 7.6), and then subjected to SDS/urea-PAGE and Western blot analysis.
4. A 100 mm × 100 mm × 1 mm gel is used routinely for the electrophoresis. The running conditions are 6–10 mA (constant current) for 12 h (overnight) or 20–25 mA for 5 h.
5. To identify the proteins involved in the crosslinking, the PVDF membranes are reused for immunoblotting with several specific antibodies.

   To separate SDS-resistant proteases, the step for removing SDS is omitted. The SDS-resistant proteases are detected as white bands at the top of the separation gel, indicating the digestion of gelatin in a blue background, as was shown previously by Sokolenko et al. ***(13)***.

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22

Thermoluminescence

A Technique for Probing Photosystem II

Prafullachandra V. Sane

Summary

The noninvasive technique of thermoluminescence (TL) monitors recombination reactions of oxidized donors and quinone acceptors of photosystem (PS)II leading to light emission. The technique is simple, inexpensive, and yet powerful in probing PSII using leaf discs, algal cells, thylakoids, or PSII preparations. The procedure for obtaining glow curves from photosynthetic material has been described. Interpretation of the obtained data requires a thorough understanding of the charge pairs responsible for generating different TL bands. This chapter discusses characteristics of different TL bands to facilitate interpretation.

Thermoluminescence and delayed light emission (DLE) are related to each other and each TL band represents one component of delayed light. The technique has found application in monitoring several aspects of the PSII electron transport. Through the possibility of interpreting changes in the redox potentials of donors and acceptors on the basis of a shift in glow peak temperature, the technique should prove useful in understanding the process of adaptation, responses to abiotic and biotic stresses, and structural modifications in the PSII polypeptides.

Key Words: Delayed light emission (DLE); electron transport; photosystem (PS)II; thermoluminescence (TL).

1. Introduction

Pre-illuminated oxygenic photosynthetic membranes emit light at distinct temperatures when they are heated from low temperature in the dark. This phenomenon is similar to the one observed in many organic and inorganic compounds and is designated as thermoluminescence (TL). Although TL has been extensively studied and used in dosimetry its study in photosynthetic membranes is rather recent. Photosynthetic TL could be defined as the emission of light at

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distinct temperatures from pre-illuminated photosynthetic membranes during warming in the dark from low temperature. The TL emitted by the oxygenic photosynthetic organisms is complex and consists of several components while the TL from anoxygenic photosynthetic bacteria is constituted by one or two components. This chapter will restrict its scope to the TL from oxygen-evolving photosynthetic membranes with occasional reference to TL in other systems. It will also not discuss the calculation of activation energy, frequency factors, and other physical parameters.

2. Historical Background

The discovery of TL in plants is attributed to Arnold ***(1)*** who previously reported delayed luminescence from chloroplasts ***(2)***. Tollin and Calvin ***(3)*** also reported TL from dried chloroplasts and followed these studies with additional reports about their characteristics. However, Arnold and colleagues studied this phenomenon in detail and made many contributions to its understanding. Arnold ***(4)*** reported TL in Chlorella cells but the instrumentation did not allow him to resolve different emission bands (peaks). He observed one band at –160°C and one peak at a higher temperature. In subsequent studies, they ***(5)*** obtained TL curves by excitation of the algal cells of *Scenedesmus* and its mutants at liquid nitrogen temperature. However the first well resolved glow curves were reported in 1969 ***(6,7)*** and it became apparent that there are several distinct TL bands emitted by the chloroplast membranes. These studies of the late sixties also provided the first evidence for the origin of TL in photosystem (PS)II. There were also indications that TL and delayed light emission (DLE) are related phenomena. The observation ***(5)*** that the mutant of *Scenedesmus* lacking PSII did not show any TL bands emitted in the temperature range of –50 to +50°C and the sensitivity of some of these bands to the addition of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] that blocks electron transport between Q_A (the primary acceptor quinone of PSII) and Q_B (the secondary acceptor quinone of PSII) were clear indications that most of the TL in this temperature range originated in PSII. The TL band observed at very low temperature (–160°C) called the Z band was unrelated to any of the photosystems (*see* **Subheading 4.1.**). These early studies were followed by detailed reports ***(8–13)*** from various laboratories ***(14,15)***. The data has elucidated the characteristics of each of the band, their relationship with the electron transport and S states of the water oxidation complex, and the charge pairs responsible for most of the peaks, for example. Initially there were some problems and confusion about the nomenclature of different bands as different groups used different system of designating the peaks. However most of the workers now use a common nomenclature. There are several excellent reviews on TL that have discussed in depth the results from different laboratories ***(14–18)***.

3. Methods

3.1. Instrumentation

Measurement of TL from photosynthetic membranes does not require a complicated or costly apparatus. In most laboratories the measurements have been made using a system assembled in the laboratory. Essentially one needs a set up with a sample holder that can be cooled and heated at controlled temperature, a temperature sensor that can measure the temperature of the sample accurately, and a red sensitive photomultiplier (PM) tube, preferably end window type with a high voltage power supply. Additionally a system that can measure and process the signals from the PM tube and temperature sensor and display on *y* and *x* axis, respectively are necessary. The signal-to-noise ratio improves with the reduction of the distance between the sample and the PM tube. The light emitted from the sample can be focused on the PM tube using a strong lens to improve the sensitivity. Furthermore, the noise can be reduced by cooling the PM tube.

With several improvements in the available electronic systems and the use of a PC with data-acquisition system, the TL set up can be rather simple and yet quite versatile. One such simple set up was described by Zeinalov ***(19)*** and has been used by the author. It is cheap to build, fast in its operation, and useful for routine observations. This system uses a small heat capacity finger (sample holder) that can be quickly cooled and heated at a fast rate (about 60°C/min). A cold finger allows measurement of several samples per hour. One of the best systems that is quite efficient and also versatile has been described by Ducruet and Miranda ***(20)***. When choosing the set up it is necessary to consider the extent of accuracy required in experiments. Most sample holders have a temperature gradient between the sample and the sensor. However, suitable corrections can be made for such a temperature gradient.

The difference in actual temperature of the sample and that monitored by the thermocouple has resulted in discrepancies in the peak temperatures reported in the literature. However it is now recognized that the temperature maximum (Tm) of the recombination (glow peak) may also vary considerably depending on the growth conditions, and the stress, if any, the organism has faced, for example.

Through the use of data-acquisition systems and ability to process the signal using software such as Origin, it is possible to bring out the signal buried in the noise. In addition, the software that enables deconvolution of the complex signal has helped identify individual peaks that constitute the complex signal. The researcher should, however, be careful in using the deconvolution programs. It should be kept in mind that only those bands that represent the known recombinations between the charge pairs such as $S_2Q_A^-$, $S_3Q_A^-$, $S_2Q_B^-$, and $S_3Q_B^-$ appearing in the temperature range of –20 and +50°C be considered as the components of the complex glow curve. Software for graphical and numerical analysis of TL that allows not only curve fitting but also calculations of activation energy values has been developed ***(20)***.

3.2. Procedures

3.2.1. Sample Preparation

The researcher may routinely use two types of samples; the leaf discs and the suspension of cells or chloroplast or thylakoid preparations. A few tips are provided for preparing the samples.

3.2.1.1. LEAF

Use leaves that are fully developed (unless the objective is to study developing or greening leaves), well exposed to light, and in an active phase of the growth. To ensure reproducibility of the data, choose leaves of the same physiological characteristics. If interested in quantitative aspects, then the area of the leaf to be seen by the PM tube should be the same and its chlorophyll concentration be known for comparison purposes. One can use the opposite pair of leaves or two halves of the same leaf for better comparison. For dark incubation, the leaf discs should be kept in well-moistened paper towels. Dark incubation may be carried out for 30 min. It should be ensured that the dark incubation has resulted in the loss of all the peaks where measurements are to be made.

In most experiments the scientists will be interested in ascertaining the effect of DCMU on the sample. This is necessary to know where the $S_2Q_B^-/S_3Q_B^-$ peaks and the $S_2Q_A^-/S_3Q_A^-$ peaks appear. In the presence of DCMU, the sample can undergo only one turnover as the electron transport beyond Q_A is blocked. To consider the distribution of S states mentioned in **Subheading 8** then DCMU treatment can provide only $S_2Q_A^-$ peak.

However, we have seen the presence of $S_3Q_A^-$ peak also in DCMU-treated samples. This could occur if some of the S_2 states do not revert to S_1 in the dark. The A_T peak that is the $His^+Q_A^-$ recombination band appears almost at the same temperature as the $S_3Q_A^-$ band (the A band) in some plants and it is difficult to differentiate the $S_3Q_A^-$ peak and A_T peak. The two bands can be distinguished by comparing the sample whose water oxidation complex has been destroyed by heat, Tris-HCl or tetranitromethane with the functional water oxidation complex.

The best way to introduce DCMU in the leaf disc is to vacuum infiltrate the solution. Routinely, use a final concentration of DCMU in the range of 10 to 20 μ*M* containing less than 2% methanol. A stock solution of 1 to 2 m*M* in 50% methanol that can be diluted 100-fold with distilled water/buffer could be used. It is necessary to apply and release the vacuum three to four times to ensure penetration of DCMU. In most cases, the properly vacuumed infiltrated leaf disc will sink in the DCMU solution.

3.2.1.2. CELLS /CHLOROPLAST SUSPENSION

When the cells/chloroplasts are used for TL measurement it is better to measure the optical density (OD) of the suspension. Usually a 5 mL suspension of 0.6 to 0.8 OD can be loaded on to a 0.45 μ filter using the vacuum filtration assembly. The chloroplast suspension cannot be loaded by vacuum filtration on a 0.45 μ filter as the size of the chloroplast is much smaller and will pass through the filter. It is advisable to use a thicker suspension of the chloroplasts or thylakoid preparation and load with the help of a brush or a pipet. DCMU at a given final concentration should be added to the suspension prior to loading the sample on the filter disc.

3.2.2. Loading the Sample

The sample used for TL measurements could be a leaf disc, a suspension of isolated chloroplasts, algal/cyanobacterial cells, or purified thylakoid preparations, for example. The sample (a leaf or a filter disc) is relaxed in the dark at room temperature for a length of time so that when measured without any illumination it does not yield any measurable TL. As expected, the TL peaks appearing at temperatures higher than room temperature would take much longer to disappear in the dark. If interested in the measurement of the peaks appearing at higher temperatures, it is necessary to relax the sample for a longer time or at a higher temperature such that it does not disturb the photosynthetic characteristics of the sample or causes distortion in the appearance of other peaks.

The relaxed sample is placed on the sample holder and positioned using a clip or some device. The use of a thin plastic sandwich type holder for positioning the leaf/filter disc is not uncommon. The use of a plastic holder does introduce a temperature gradient but this can be corrected. The plastic holder protects the temperature sensor of the holder. The sample is then subjected to illumination at a given temperature or in a range of temperatures (*see* **Subheading 3.2.3.**) and quickly cooled by dipping in liquid nitrogen. The sample holder is then positioned in a transparent Dewar flask so that light emitted during warming of the sample is focused on the window of the photomultiplier tube. The sample is then heated at a predetermined rate. Most studies use a heating rate of 0.5 to 1°C/s.

3.2.3 Excitation of the Sample

Earlier studies ***(10–12)*** used continuous illumination from room temperature to liquid nitrogen temperature while several studies used illumination at a fixed temperature. Although the light intensity used in continuous illumination is not critical it should be around the intensity that the organism was experiencing during its growth. If continuous illumination during freezing to low temperature is being used then the rate of cooling of the sample must be the same each time to obtain reproducible data. The exposure of the sample to one, two, or multiple flashes at a desirable temperature generates selective peaks *(21)* identified to be arising in a given recombination reaction. In recent studies, flash excitation has been preferred for this purpose. The flash must be intense and of short duration to ensure only a single turn-over.

Although flash excitation generates specific charges on donors and acceptors that on heating yield TL, the continuous illumination generates a complex glow curve. It is, however, possible to separate out different peaks constituting the complex signal and sometimes provides useful information *(22)*.

Scientists have also used excitation by flashes/illumination at two different temperatures *(14)* and excitation at room temperature followed by low temperature. The choice of excitation regime depends upon the objectives of the study. The flashing by one or a series of flashes is usually done at around 0°C. For this purpose, the dark relaxed sample should be slowly cooled to the desired temperature. The sample immediately after the flash should be quickly cooled to –50°C or lower. It should be realized that the recombination of charge pairs $S_2Q_A^-$, $S_3Q_A^-$, $S_2Q_B^-$ and $S_3Q_B^-$ starts immediately. Peaks that appear at lower temperatures such as $S_3Q_A^-$ and $S_2Q_A^-$ will disappear rather fast. Incidentally, one can use this relaxation of the "S" states for measuring their stability using different time periods before cooling the sample to low temperature.

4. Interpretation and Theoretical Considerations

Unlike many other techniques, the interpretation of the TL data requires an understanding of the characteristics of different TL bands and their origin in the charge pairs responsible for their appearance. Although the temperature at which a given peak appears is indicative of which charge pair recombination it may be representing it has to be ascertained by flash/or inhibitor studies. The researcher, therefore, must have a good understanding of what characteristics each peak has. He or she must also know how each charge pair is generated depending on the illumination regime used by him/her. In this section a brief description is provided.

4.1. Characteristics of Important TL Bands

There is unanimity in the nomenclature of different TL bands and **Table 1** provides the Tm of different bands normally observed in photosynthetic material, the identified recombining species (charge pairs) for their emission, and the related DLE component, whenever known. Among the bands observed, those that appear at low temperatures (liquid helium temperatures; *see* ref. ***23***) and the Z band that appears at −160°C are of little importance in probing the functioning of PSII. They appear in isolated chlorophylls and the light-induced charge separation associated with either PSII or PSI is not essential. Similarly the

Table 1
Thermoluminescence Peak Temperatures (Tm), Charge Pairs Responsible for Their Emission, and Related DLE Components

Peak	Approximate Tm °C	Charge pairs	Related DLE component
Very low temp peaks	–250, –220, –200	Originate in aggregated chlorophylls	
Z peak	−160	Chl^+Chl^-	
PSII related bands			
Zv (variable)	–80 to –30	$P680^+Q_A^-$	approx 150 µs
A band	−10	$S_3Q_A^-$	4 ms
A_T band	−10	$His^+Q_A^-$	4 ms?
Q band	+ 5	$S_2Q_A^-$	2 s
B1 band	+20	$S_3Q_B^-$	30 s
B2 band	+30	$S_2Q_B^-$	60 s
C band	+50	$TyrD+Q_A^-$	10 min
High temp bands	50 to 70	Oxidative chemiluminescence	

peaks appearing at high temperatures (usually higher than 50°C) are also not relevant for probing PSII or PSI. The most important TL bands that provide information on PSII occur between the temperature range –50 to +50°C. A brief discussion on their origin is given below to enable the researcher to interpret the obtained data. For a detailed discussion, readers are requested to refer to earlier reviews on TL ***(14–18)***.

1. The A band: This band appearing at about –10°C arises in a recombination reaction between S_3 and Q_A^-. The most reliable evidence ***(24)*** suggests its origin in $S_3Q_A^-$. It was demonstrated that when a dark-adapted sample was first exposed to two flashes followed by continuous illumination at liquid nitrogen temperature a band appeared at –10°C. The origin of this band in S_3 state on the basis of flash excitation was earlier suggested ***(25)***. It was proposed ***(24)*** that excitation by two flashes at room temperature generates S_3 state, which is stabilized at low temperature. If the sample is further illuminated at low temperature then reduction of Q_A occurs by the oxidation of cytochrome b559. The S_3 and Q_A^- recombine on warming producing the A band. Because the temperature at which the A band appears is similar to the one at which the A_T band appears there has been some confusion in the characteristics of the peak appearing at –10°C.
2. The A_T band: A peak appearing at –10°C (but different than A band mentioned above) has been shown to be unaffected by inhibitors of water oxidizing complex ***(26–28)*** but is present in PSII fraction ***(8,12,13)***. Thus PSII is essential but S states are not participating in the production of this band. This band has been proposed to

arise in His^+ of the PSII reaction center protein and Q_A^- ***(29)***. Using site-directed mutagenesis of the histidine on the donor side of PSII D1 polypeptide, it was demonstrated ***(30)*** that the emission of the A_T band is affected by substitution of His 195 and His 190 in *Chlamydomonas*.

3. The Q band: This is a well-characterized band that appears/intensified in DCMU treated chloroplasts/leaf discs/algal cells ***(7,8,11,13)***. It was demonstrated ***(11)*** that the appearance of this peak is accompanied by the decrease in the fluorescence yield showing that the oxidation of Q_A^- is associated with its appearance. This peak appears if DCMU-treated chloroplasts are exposed to a single flash at –15°C ***(31)***. Its appearance requires functional water oxidizing complex since the peak was sensitive to heat treatment ***(12)*** and it was lost in Tris-HCl treated or tetranitromethane treated chloroplasts ***(32)***. Its origin in $S_2Q_A^-$ recombination is thus clear. This is an important peak as its Tm reflects on the redox characteristics of Q_A ***(22)***. There are several references relating to this peak in the literature.
4. The B bands: The most studied band in photosynthetic TL is the B band. Earlier studies using continuous light had shown that the B band appearing around 30°C is lost by the addition of DCMU ***(5,7,11,13)***. The B band is absent if the oxygen evolving complex is either absent or inhibited ***(27,28,33)*** suggesting that it is related to the S states. The flash data indicated that it oscillates with a period of four ***(21,34)***. Because the flash-induced TL maximum appeared at second and sixth flash it was logical to conclude that S_3 state may be responsible. However, B band also appears if a relaxed sample is excited by a single flash. Thus, the B band was suggested to result from the S_2/S_3 state. Because the peak was sensitive to DCMU, the negative charge appeared to be coming from Q_B^-. A number of observations have confirmed the involvement of Q_B^- in the appearance of this peak ***(15,17,18)***. Earlier, most workers considered the origin of the B band in $S_2/S_3Q_B^-$ recombination. However, it is now clear that there are two separate bands one arising in $S_2Q_B^-$ and another appearing at a lower temperature (by about 10–15°C) than the $S_2Q_B^-$ band and is the result of the $S_3Q_B^-$ recombination. In leaves of different species, we (unpublished data) see clearly $S_2Q_B^-$ band by exciting the dark relaxed sample with one flash and a combination of two peaks after two flashes originating in $S_2Q_B^-$ and $S_3Q_B^-$, both fully sensitive to DCMU. The $S_3Q_B^-$ recombination has been shown to emit 1.7- to 2-fold higher intensity than $S_2Q_B^-$ recombination ***(35,36)***.
5. The C band: A band that appears at around 50°C was reported in earlier studies ***(7,11)***. It is enhanced by DCMU ***(11,13)*** and oscillates with flash excitation giving maximum in the S_0 and S_1 state ***(37)***. These observations suggest that the negative charge may come from Q_A^- while the positive charge may reside on some donor component of PSII. The earlier suggestion ***(37)*** that S_0/S_1 states may be involved could not be confirmed. It has been suggested that the positive equivalents located on YD^+ (the tyrosine of D2 polypeptide) may recombine with Q_A^- to yield this band ***(38–40)***. This band was shown to be preferentially excited by far red (740 nm) light and enriched in a PSI fraction ***(12)***.

More recently, the band appearing at 45–50°C that shows oscillations and can also be enhanced by far-red light has been suggested to originate in PSII centers that were initially in S_2/S_3Q_B oxidation state. During warming, the Q_B in these centers may become reduced by reversed electron flow or reduction of PQ pool via an NADPH/PQ oxidoreductase ***(41,42)***. In addition, it was suggested that the intensity of this band may reflect the energy status of chloroplasts.

6. The Zv band. This band is designated as Zv band because its emission temperature varies between –80 and –30°C depending on the temperature of excitation ***(13)***. A mechanism suggesting that the Zv band arises from charging of Z band and A band was proposed ***(43)***. The charge pair responsible for its origin has been suggested to be $P680^+Q_A^-$ ***(44,45)***. Using PSII reaction center preparations the band has been assigned to oxidized tyrosine and reduced plastoquinoine ***(46)***.

The peak temperatures are indicative of a given recombination reaction. However the temperature maximum may vary considerably depending upon the conditions of growth, excitation stress, abiotic stresses, increase or decrease of energetic potential (NADPH + ATP), for example. In wild type Synechosystis sp PCC 6803 grown at 30°C, $S_2Q_B^-$ was observed at 24–26°C while the $S_2Q_A^-$ was at 16–18°C ***(47)***. In its mutants psbV and psbU the $S_2Q_B^-$ appeared at 30–32°C and $S_2Q_A^-$ at 16–18°C. For the same Synechosystis sp PCC 6803 grown also at 30°C the S_2Q_B- was reported at 36°C ***(48)*** although in its NDFS mutant it appeared at 28°C. In Synechococcus grown at 36°C, $S_2Q_B^-$ was at 37°C while the same peak appeared at 30°C when the organism was shifted to 25°C that induces exchange of D1/1 by D1/2 ***(22)***. Therefore, it is necessary to ascertain the charge pair that a peak represents by some other way. Routinely, the effect of DCMU, the peak position with a single flash and two flashes are studied for those peaks that relate to $S_2Q_B^-/S_3Q_B^-/S_2Q_A^-$ peaks. Although for the low temperature –10°C band it will be necessary to ascertain whether the band observed is A band ($S_3Q_A^-$) or A_T band ($His^+Q_A^-$) by inactivating the water oxidizing complex using tetranitromethane or Tris-HCl or heat treatment if one is dealing with a suspension of cells or thylakoid/chloroplast preparation.

4.2. Origin of Thermoluminescence

In semiconductors the TL has been well characterized and its origin has been demonstrated to be in a recombination of a free hole and a free electron generated during irradiation of the sample by electromagnetic radiation. Initially, this mechanism was proposed to be responsible for TL in photosynthetic membranes ***(49)***. However, soon it was realized that in oxygen evolving photosynthetic membranes the charges responsible for TL arose within PSII ***(5)***.

The observation that glow curves consisted of several peaks had already suggested that different charge pairs were involved in generating different glow peaks. The observations ***(34)*** that the major TL bands exhibited period four oscillations provided strong evidence for the participation and specific identification of S states of the water oxidizing complex for providing positive charges for the recombination reaction resulting in TL. The earlier studies ***(11,12)*** showing loss of most of the TL bands when S states were destroyed by Tris-HCl washing or by tetranitromethane had indicated the requirement of the functional water oxidizing complex for generating TL bands. The use of flashes in inducing specific TL bands has proven to be valuable in application of TL in probing PSII reactions.

Although the generation of positive charges by the water-oxidizing complex identified the S states primarily S_2 and S_3 in TL, the use of inhibitors on the acceptor side identified the components providing the negative charges for recombining with the positive charges. It was already evident from DCMU studies *(7)* that the acceptor side was involved in TL. Desai et al. *(11)* provided the first evidence for linking TL to the oxidation of the fluorescence quencher (now identified as Q_A). They showed that the decrease in fluorescence yield coincided with the appearance of one of the TL peaks (Q-band). It is now well established that the major TL bands appearing between −10°C and +50°C are associated with the recombination of Q_A^- and Q_B^- with the S_2 and S_3 states of the water oxidizing complex. There are other recombinations involving other donors and quinone acceptors (Q_A/Q_B) of PSII that also yield TL peaks as mentioned earlier.

The light emitted as TL has characteristics similar to the chlorophyll fluorescence. Therefore, it was clear that the final step leading to TL must be the excitation of chlorophyll to its singlet state. Those peaks that arise in isolated chlorophylls (Z band, low temperature bands and the high temperature bands) probably originate in Chl^+Chl^- recombination. Those peaks that require a charge separation in PSII (the Z_v, A, A_T, Q, B, and C bands) originate as a result of final recombination of $P680^+Ph^-$ (P680 is the reaction center chlorophyll of PSII and Ph is the pheophytin, the primary electron acceptor of PSII) leading to the formation of singlet excited P680. This singlet excited P680 could emit light as TL or the recombination could excite a singlet in the coupled antenna chlorophyll that will emit light with the characteristics of chlorophyll fluorescence. The illumination of photosynthetic membranes results in charge separation ($P680^+Ph^-$) that is quickly stabilized by the oxidation of donor D to D^+ and the reduction of acceptor A to A^-. In PSII, the donor to P680 is tyrosine 161 of D1 polypeptide, which reduces P680 and extracts an electron from water-oxidizing complex generating the higher oxidized S state in the process. The acceptor in PSII is Q_A (the bound quinone) that oxidizes Ph^- and is reduced to Q_A^-. It immediately reduces the mobile quinone Q_B unless the electron transport between Q_A and Q_B has been blocked. Thus on the acceptor side Q_A^- and or Q_B^- is generated. The formation of D^+A^- charge pair is accompanied by the loss of energy (the stabilization energy) and its recombination is prevented because of the barrier of the activation energy. Further lowering the temperature of the sample prevents the recombination of D^+A^-. This stabilized charge pair (positively charged S states and the negatively charged quinone acceptors) recombines when the temperature of the sample is raised during the warming of the sample for TL measurement. The D^+A^- recombination occurs through a series of back reactions finally resulting in the generation of $P680^+Ph^-$. The recombination of this radical pair has sufficient energy to generate an excited singlet of chlorophyll.

The increase in the temperature of a given TL peak indicates that the activation energy needed to permit recombination is more. Conversely the decrease in the peak temperature indicates that the activation energy needed for the recombination has been lowered. As a rule of thumb the increase or decrease in peak temperature is related to the change in the redox potential of the positively or negatively charged species. Thus if a given treatment causes a shift in the peak temperature it is possible to conclude that the treatment has changed the redox potential of either the acceptor or the donor species. Although the redox potential *per se* of the donor or acceptor cannot be calculated from the glow curve, it is possible to calculate the free energy of detrapping. From this one can infer about the extent of a change in the redox potentials of the donor and acceptor species.

The fact that important TL bands that reflect on the functional aspect of PSII originate in a recombination of positively charged donors (S states) and negatively charged acceptors Q_A^- and Q_B^- allows one to explain the oscillatory behavior of TL bands and prediction of luminescence pattern on flash excitation. Each flash generating $P680^+Ph^-$ causes oxidation of the Mn cluster of water oxidation complex. Thus beginning with S_0 the flash excitation in sequence converts S_0 to S_1, S_2, S_3 and S_4 depositing one positive charge through removal of an electron. However, simultaneously there is a release of proton in a 1,0,1,2 pattern. Thus, when S_0 is converted to S_1, a proton along with an electron is lost resulting in no net positive charge on S_1. The conversion of S_1 to S_2 with a loss of an electron is not accompanied by a loss of a proton and hence S_2 carries a net positive charge. The conversion of S_2 to S_3 through a loss of an electron is accompanied by a loss of a proton. Thus, S_3 retains a positive charge. The S_3 to S_4 conversion through a loss of an electron is accompanied by a loss of two protons. The S_4 is not stable and quickly returns to S_0 through the evolution of oxygen from water. Thus, the only positively charged S states are S_2 and S_3 and predictably they recombine with the negatively charged quinone acceptors Q_A^- and Q_B^-.

Just as donor is oxidized by light induced charge separation $P680^+Ph^-$ the acceptor side is reduced. Ph^- is stabilized by reduction of Q_A to Q_A^-. However Q_A^- immediately reduces Q_B to Q_B^- depositing a negative charge on Q_B. If Q_B was already in a reduced state then Q_B^- will be converted to Q_B^{2-}. It will be immediately protonated and replaced by another PQ (plastoquinone) molecule.

Thus, depending on the proportion of Q_B to Q_B^- flash excitation will produce positively charged S_2/S_3 and negatively charged Q_B^-. Inoue ***(17)*** has explained the flash induced $S_2/S_3Q_B^-$ band by assuming different initial distribution of S_0/S_1 and Q_B/Q_B^-. The details are presented as notes at the end of the chapter.

5. Relationship Between TL and DLE

As mentioned earlier, the discovery of TL had its origin in the observation of delayed luminescence from chloroplasts ***(2)***. Shuvalov and Litvin ***(6)*** attempted to establish the relationship of different phases of delayed fluorescence with different TL bands. Flash studies had shown that both TL and DLE undergo oscillations. The identification of specific charge pairs namely $S_2/S_3Q_B^-$ and $S_2/S_3Q_A^-$ responsible for different TL bands and the estimated half life of each of the TL band at 25°C running into seconds ***(9,50,51)*** suggested that different components of DLE may be related to different TL bands.

Subsequently, it was demonstrated that delayed fluorescence in fact mimics TL and that the two are interconvertible ***(52)***. This suggested a common origin of TL and DLE. Subsequent work ***(53,54)*** provided additional support for the relation between TL and DLE. Using different inhibitors of PSII electron transport that affect both TL and DLE, a correlation between each of the component of DLE with TL bands was established ***(55)***. It must be realized that some components of DLE may not have any relation with the TL bands as they may arise in temperature independent processes ***(18)***. The temperature jump experiments that show induced DLE by quickly raising the temperature of pre-illuminated samples also establish the correlation between DLE and TL ***(56–58)***.

The advantage of measuring TL over DLE under some situations is that the components of DLE are better resolved using TL as each separate peak corresponds with one or the other component of DLE.

6. Application in PSII Research

The noninvasive TL technique has found several applications in probing PSII. Because it monitors recombinations in charge pairs involving stable oxidized S states and reduced Q_A and Q_B, the technique has a potential to detect structural changes in both the donor and acceptor side of PSII. The change in glow peak temperature reflects the changes in redox characteristics of Q_A and Q_B on the one hand and the S_2 and S_3 states on the other. Therefore it permits detection of subtle changes in the electron transport within PSII, stability of S states, and perturbation in the rate constants, for examples. A list of some of these with one or two relevant references is given below:

1. Effect of herbicides that block electron transport between Q_A and Q_B ***(59)***.
2. Study of herbicide resistance ***(60)***.

3. Site-directed mutations in D1 and D2 relating to donor and acceptor side ***(61,62)***.
4. Study of transitions/stability/deactivation of S states ***(21,63,64)***.
5. Bicarbonate effects on PSII ***(65,66)***.
6. Effects of removal and addition of extrinsic proteins on PSII preparations ***(67)***.
7. Effects of Ca and Cl depletion on PSII ***(68,69)***.
8. Light and temperature stress ***(22)***.
9. Seasonal changes in the activity/activation of PSII ***(70)***.
10. Acclimation ***(71)***.
11. Characterization of PSII reaction center ***(72)***.
12. Role of lipids for structural stability of Q_B ***(73)***.
13. Changes in energetic potential (NADPH + ATP) in plants ***(42)*** and metabolic state of leaf ***(41)***.

7. Concluding Remarks

The technique of TL is rather simple, cheap, easy and yet it monitors in vivo changes in redox characteristics of the donors (S states) and acceptors (Q_A and Q_B) that reflect on the changes in their structural environment. It has therefore found increasing usefulness in several areas of PSII research. Although one can calculate activation energies (not discussed in this chapter) from the glow curves ***(51,74)*** it is not possible to calculate directly the redox potentials.

8. Notes

8.1. Identification of Precise Charge Pairs

The most useful charge pairs yielding valuable information on the functioning of PSII are $S_3Q_A^-$, $S_2Q_A^-$, $S_3Q_B^-$ and $S_2Q_B^-$. For the luminescence to occur it is necessary that the donor species have a positive charge while the acceptor species must have the negative charge. Among the S states only S_2 and S_3 states are the positively charged stable S states based on the proton release pattern of 1, 0, 1, 2. Thus although each flash extracts one electron from the S state, the proton release pattern results in positive charge only on S_2 and S_3. It is possible to predict luminescence involving $S_2Q_B^-$ and $S_3Q_B^-$ recombinations on the basis of flash induced redox reactions within PSII. Inoue ***(17)*** has provided a detailed discussion of the same.

When the photosynthetic membranes/cells/leaf tissue/chloroplasts are relaxed in the dark for a long time, TL is not observed. Under such a dark-adapted state the only S states observed are S_0 and S_1. The S_2 and S_3 states are deactivated in the dark to S_0 and S_1 states. The proportion of $S_0 : S_1$ is approximately 25 / 75. The proportion of $Q_B^- : Q_B$ under similar conditions is also 25 / 75. Thus in the dark-adapted sample we have the following distribution of non-luminescent combination of charge pairs.

$S_0Q_B : S_0Q_B^- : S_1Q_B : S_1Q_B^-$ ($S_0 : S_1 = 2 : 6$ and $Q_B^- : Q_B = 2 : 6$)

1/1/5/1

A short duration intense flash will convert the distribution of the above to the following

$S_1Q_B^- : S_1Q_B : S_2Q_B^- : S_2Q_B$

1/1/5/1

Out of these, only $S_2Q_B^-$ is luminescent yielding five units of TL intensity.

However if the dark relaxed sample was exposed to two successive short duration flashes the distribution of charge pairs would be as follows

$S_2Q_B : S_2Q_B^- : S_3Q_B : S_3Q_B^-$

1/1/5/1

Out of these, $S_2Q_B^-$ and $S_3Q_B^-$ would be luminescent and hence would yield two units of TL intensity. However actual intensity observed from $S_3Q_B^-$ is approx 1.7-to 2-fold higher than $S_2Q_B^-$. Thus we should get one unit of TL from $S_2Q_B^-$ and about 1.7 units of TL from $S_3Q_B^-$ yielding a total intensity of about 2.7 units or almost half that one gets after one flash.

If the dark relaxed sample is exposed to three successive short duration flashes the distribution of the charge pairs will be

$S_3Q_B^- : S_3Q_B : S_0Q_B^- : S_0Q_B$

1/1/5/1

Only $S_3Q_B^-$ will yield luminescence equivalent to about 1.7 unit of TL intensity.

If the dark relaxed sample was exposed to four successive short duration flashes the distribution of charge pairs will be identical to what existed in the dark. Thus there will be no luminescence after four flashes.

These theoretical predictions have been observed ***(17)*** but are not seen all the time since misses and double hits do occur and the distribution of Q_B to Q_B^- could also be different in different samples. The predicted luminescence is a good guide and indicates:

1. To see primarily the $S_2Q_B^-$ peak, expose the dark relaxed sample to only one flash.
2. To see only $S_3Q_B^-$ peak expose the relaxed sample to three flashes.
3. Exposure to two flashes will yield luminescence from both $S_2Q_B^-$ and $S_3Q_B^-$. These two peaks can be easily separated by deconvolution. A single flash provides a major $S_2Q_B^-$ band with little contamination from $S_3Q_B^-$ while two flashes yield a peak of $S_3Q_B^-$ with a shoulder of $S_2Q_B^-$ appearing at a slightly higher temperature (unpublished data).

The predicted distribution of $S_2/S_3Q_B^-$ depends upon how well relaxed the sample is in the dark. If the sample is a thylakoid preparation and has been dark adapted for a short period then the distribution of the S states and $Q_B^- : Q_B$ has been observed to be $S_0 : S_1 = 25 / 75$ but $Q_B^- : Q_B$ is 50 / 50. Thus the briefly dark adapted sample will have the following distribution

$S_0Q_B : S_0Q_B^- : S_1Q_B : S_1Q_B^-$

1/1/3/3

None of the above charge pairs are luminescent. One can predict the distribution of charge pairs on exposure to 1, 2, 3, and 4 short duration flashes. The distribution will be as follows:

$S_0Q_B : S_0Q_B^- : S_1Q_B : S_1Q_B^-$

1/1/3/3 No Flash (No TL)

$S_1Q_B^- : S_1Q_B : S_2Q_B^- : S_2Q_B$

1/1/3/3 One flash (3 TL Units)

$S_2Q_B : S_2Q_B^- : S_3Q_B : S_3Q_B^-$

1/1/3/3 Two flashes (6.1 TL Units)

$S_3Q_B^- : S_3Q_B : S_0Q_B^- : S_0Q_B$

1/1/3/3 Three flashes (1.7 TL Units)

After four flashes the distribution of S states will be the same as existed in the dark relaxed sample. Thus in this type of sample the flash induced TL intensity will show a peak at second, sixth, for example, flash numbers. In addition, a single flash will yield only $S_2Q_B^-$ peak while the two flashes will give a major peak of $S_3Q_B^-$ with a shoulder of $S_2Q_B^-$ appearing at a slightly higher temperature. Therefore, irrespective of the type of relaxed sample, a single flash will always give an $S_2Q_B^-$ peak while the two flashes will give a mixture of both $S_2Q_B^-$ and $S_3Q_B^-$ with a higher intensity of $S_3Q_B^-$.

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23

Detection of Free Radicals and Reactive Oxygen Species

Éva Hideg

Summary

In plants, reactive oxygen species (ROS), also known as active oxygen species (AOS), are associated with normal, physiological processes as well as with responses to adverse conditions. ROS are associated with stress in many ways; as primary elicitors, as products and propagators of oxidative damage, or as signal molecules initiating defense or adaptation. The photosynthetic electron transport is a major site of oxidative stress by visible or ultraviolet light, high or low temperature, pollutants, or herbicides. ROS production can be presumed from detecting oxidatively damaged lipids, proteins, or pigments as well as from the alleviating effects of added antioxidants. On the other hand, measuring ROS by special sensor molecules provides more direct information. This chapter focuses on the application of spin trapping electron paramagnetic resonance (EPR) spectroscopy for detecting ROS; singlet oxygen and oxygen free radicals in thylakoid membrane preparations.

Key Words: EPR spectroscopy; free radical; reactive oxygen species (ROS); singlet oxygen; spin trapping; thylakoid.

1. Introduction

A free radical is defined as any species containing one or more unpaired electrons *(1)*. Oxygen radicals are an important group among free radicals, although carbon- or sulphur-centered radicals and free radical oxides of nitrogen are also formed in living systems. Besides oxygen-centered radicals, the group of reactive oxygen species (ROS) also involves nonradical forms of oxygen, such as singlet oxygen or hydrogen peroxide. Although the collective terms "reactive species," ROS, and free radical are frequently used as synonyms, the groups of chemical molecules they refer to are not identical **(Fig. 1)**. All ROS are derivatives of molecular oxygen **(Fig. 2)**. Superoxide is formed

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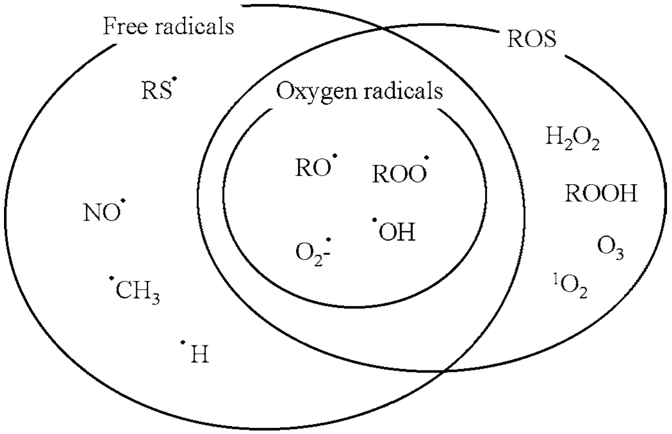

Fig. 1. Chemical classification of "reactive species". Examples of oxygen free radicals: superoxide ($O_2^{\bullet-}$), hydroxyl ($^\bullet$ OH), peroxyl ($^\bullet$ OOR) and alkoxyl ($^\bullet$ OR) radicals. Examples of nonradical ROS: singlet oxygen ($^1O_2$), oxone (O_3), hydrogen-peroxide (H_2O_2), and other peroxides (ROOH). Free radicals that are not oxygen-centered are represented by sulphur-centered thiyl (RS $^\bullet$), carbon-centered methyl ($^\bullet$ CH_3) radicals and the hydrogen atom (H $^\bullet$), which is the simplest free radical. Certain oxides of nitrogen, such as NO $^\bullet$ are also free radicals.

when one electron is added to the ground-state oxygen molecule, addition of one more electron followed by protonation results in H_2O_2. Further reductions and protonations convert H_2O_2 into the neutral form, H_2O. Singlet oxygen is also generated from molecular oxygen, but via energy transfer. In this reaction, the spin restriction of ground (triplet) state oxygen is removed, so the oxidizing ability is increased ***(1–3)***.

Plants, like any other living organism, constantly produce and extinguish ROS. Under natural conditions, there is usually no detectable pool of reactive species, because these react with other molecules shortly after being produced. Some of these reactions are harmful, leading to oxidative modification of membrane lipids, proteins or DNA; although others, for example the controlled cycling of electrons in the Asada-Halliwell cycle ***(4)***, or the H_2O_2 dependent polymerization of phenols in lignin formation ***(1,5)*** are elements of ordinary plant metabolism. A complex system of enzymes and other ROS scavenging molecules keep the concentration of free radicals low, contrary to continuous production ***(5–7)***. This balance may be tipped under stress conditions, by increased radical production, or by decreased antioxidant capacity. ROS production may increase as a result of imperfect electron transport, for example,

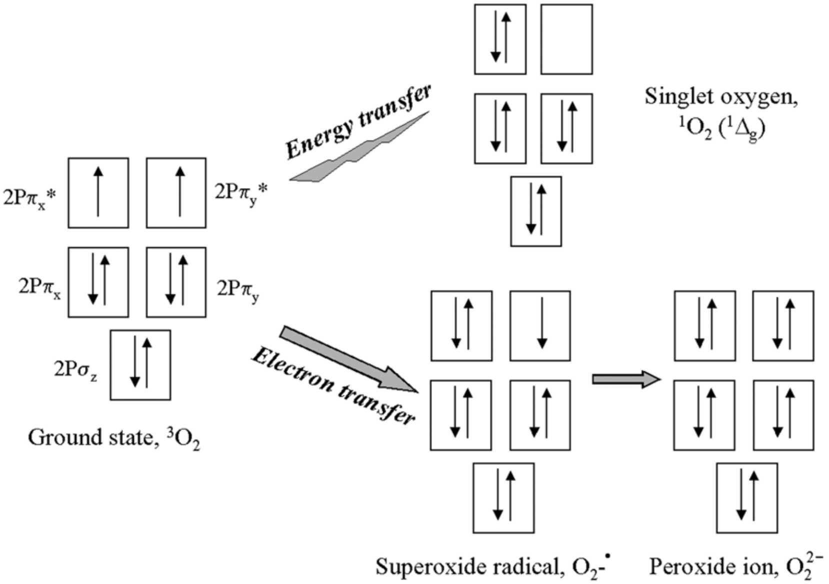

Fig. 2. Ground state oxygen and its derivatives. Simplified illustration of electrons on molecular orbits.

in chloroplasts or in mitochondria, by electron leakage or via energy transfer to oxygen. On the other hand, external sources (e.g., ozone, photosensitizers, or bipyridyl herbicides) may also induce free radical production in high concentrations at unexpected locations, as compared to normal metabolic reactions. Scavenging these radicals may exhaust the antioxidant system, for example by oxidizing the ascorbate and glutahione pools, if these cannot be restored (re-reduced) fast enough. In addition, adverse temperature or water conditions may decrease the activity of antioxidant enzymes, so these are unable to process the increased load of free radicals. The photosynthetic electron transport is a major site of oxidative stress by visible or ultraviolet light, high or low temperature, pollutants, or herbicides ***(7–10)***.

This chapter focuses on detecting radicals under conditions, when the imbalance in the production and scavenging of reactive species allows specific external probes (ROS sensor molecules) to compete with the natural targets for ROS. Our examples are taken from research on ROS, singlet oxygen, and free radical production in plants under stress by excess photosynthetically active radiation (photoinhibition). With minor adaptations, these techniques can be applied to study other conditions, when increased production of ROS is expected.

2. Materials

1. Grinding medium: Mes 50 m*M*, pH 7.2, 0.4 *M* sucrose, 15 m*M* NaCl, 5 m*M* $MgCl_2$, and 5 m*M* ascorbate.
2. Resuspension buffer: potassium phosphate 50 m*M*, pH 7.2, 0.2 *M* sucrose, 15 m*M* NaCl, and 5 m*M* $MgCl_2$.
3. DMPO (5,5-dimethyl-pyrrolin *N*-oxide).
4. Tiron (1,2-dihydroxybenzene-3,5-disulphonate).

3. Methods

3.1. Thylakoid Membranes Isolation

1. Isolate thylakoid membranes from market spinach. Wash mature and healthy leaves in tap water, dry, and remove of midveins.
2. Chop the leaves in a blender with ice cold grinding medium. Use approx120 mL of grinding medium for 200 g of leaf and grind in short, 10- to 12-s intervals, repeated five to six times.
3. Filter the mixture through eight layers of cheesecloth and centrifuge at 7000*g* for 15 min.
4. Resuspend the pellet in resuspension buffer to approximately one-third of original volume.
5. Centrifuge at 300*g* for 5 min to remove crude pellet and possible debris.
6. Centrifuge the supernatant again at 7000*g* for 15 min, and resuspend the pellet in the resuspension buffer to approx 1200–1500 μg Chl/mL.
7. Conduct all centrifugation steps at 5–7°C, resuspend while keeping centrifuge tubes on ice. Store thylakoid preparations in 0.3–0.5 mL volumes at –80 °C until use.
8. Once thawed, keep samples on ice in the dark until use and dilute to 60–200 μg Chl/mL using the above resuspensions buffer.
9. Add ROS traps to diluted thylakoids in concentrations indicated below.
10. Once thawed, either discard surplus thylakoids or freeze again for experiments other than ROS detection.

Experiments with samples prepared in sodium-phosphate buffer gave similar results, but using Hepes lowered the amount of trapped ROS, although Hepes had no influence on other parameters, such as the extent of stress induced damage. Photosynthetic activity of the preparations should be checked, for example by measuring steady-state oxygen evolution with a Clark-type oxygen electrode in the presence of an electron acceptor.

3.2. Spin Trapping EPR Spectroscopy

Electron paramagnetic resonance (EPR), also called electron spin resonance (ESR) spectroscopy, detects the presence of unpaired electrons, providing an excellent technique for measuring free radicals. However, because free radicals are highly reactive and are usually present in small concentrations, their direct detection is practically impossible at room temperature, unless their radi-

Spin trap	+	**ROS**	→	**Spin adduct**
diamagnetic		paramagnetic		paramagnetic
stable		$10^{-6} - 10^{-1}$ sec		min - hour

Fig. 3. Representation of the principle of spin trapping indicating magnetic features and approximate lifetimes of the reagents and the product.

cal nature is stabilized by reacting with trapping molecules *(12,13)*. The spin adducts produced in reactions between the free radical and the spin trap are also radicals but are reasonably stable for biological experiments **(Fig. 3.)**. Although singlet oxygen is not a free radical form of ROS, its reaction with specific traps may also yield EPR detectable nitroxide radicals, therefore $^1O_2$ detection is also discussed here. Structures of spin traps applied in this study are shown in **Figs. 4–6**. These chemicals are commercially available from several companies.

EPR spectra of spin adducts are usually measured at room temperature, using X-band spectrometers. In our experiments, a Bruker ECS-106 machine was used. EPR parameters were: 9.45 GHz microwave frequency, 12 mW microwave power, 100 kHz modulation frequency, 0.2 mT modulation amplitude, 10^4–10^5 receiver gain, depending on signal intensities and 10.2 ms time constant. To improve the signal : noise ratio, nine spectra were averaged.

Samples were measured in glass capillaries, at 15–20 µL volumes. Because functioning plant samples are usually in the water-based preparation buffers, a critical step of EPR measurements is using as much sample as possible, but preventing interference from the high microwave absorbance of water. Optimal amounts may vary with the type and quality of the spectrometer, especially by its cavity.

Using an EPR flow cell allows the measurements of larger volumes than in capillaries or sample tubes, but the flow cell needs rigorous cleaning between changing samples in order to avoid artifacts.

When using disposable capillaries, comparative EPR quantitation should be assured by keeping identical experimental conditions regarding both the samples (volume, concentration) and sample holders (uniform tubes, the same central position in the cavity).

3.3. Applications of Spin Traps in Photosynthetic Membrane Preparations

The following examples were taken from studies on ROS in the oxidative damage of isolated thylakoid membranes caused by excess photosynthetically

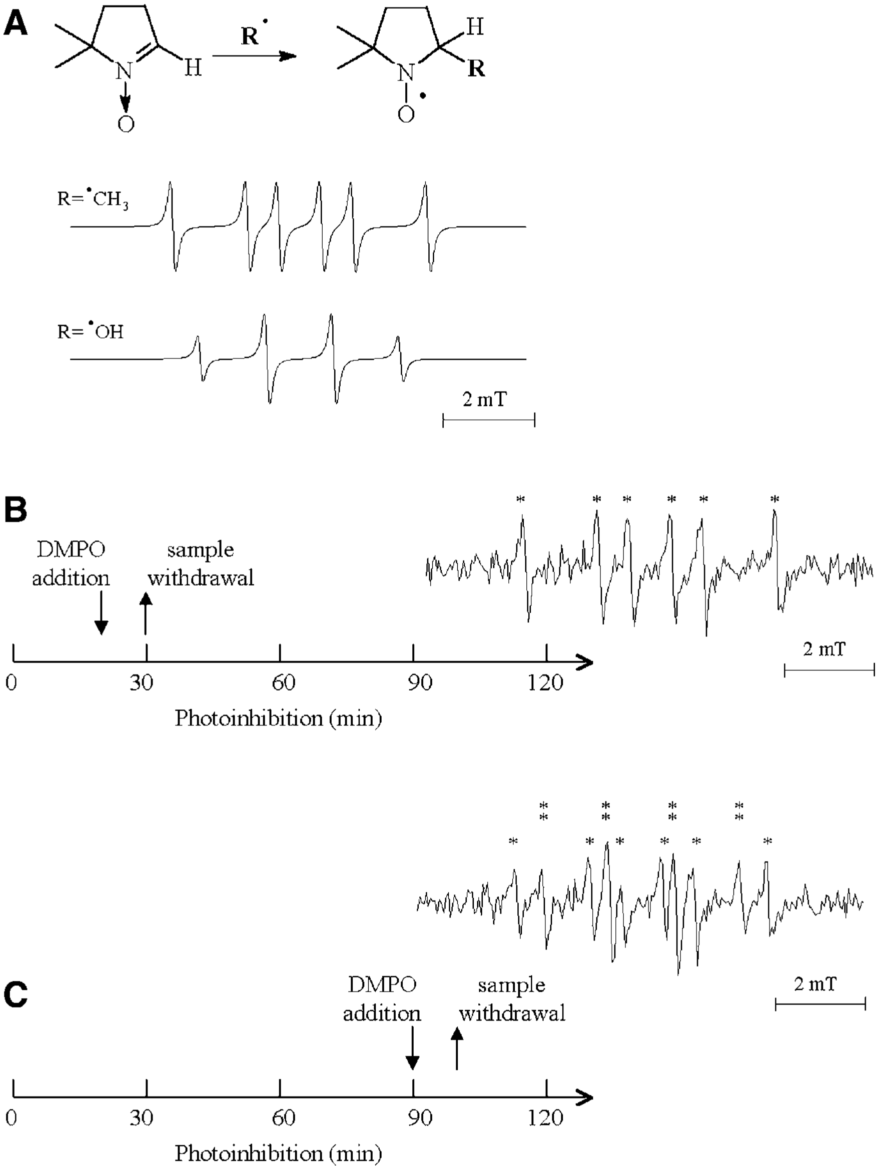

Fig. 4. Spin trapping experiment using DMPO (50 m*M*) in isolated thylakoid membranes (100 µg/mL) exposed to 1600 µ*M* m^{-2} s^{-1} photosynthetically active radiation. **(A)** structure of DMPO and the radical adduct formed upon reaction with a free radical represented by $R^\bullet$. Depending on the chemical nature of $R^\bullet$, radical adducts show EPR spectra characterized with different hyperline splitting. **(B)** and **(C)** sampling strategy and typical EPR spectra. Single asterisks indicate the six lines belonging to a carbon-centered radical adduct, double asterisks identify the four lines of DMPO-OH.

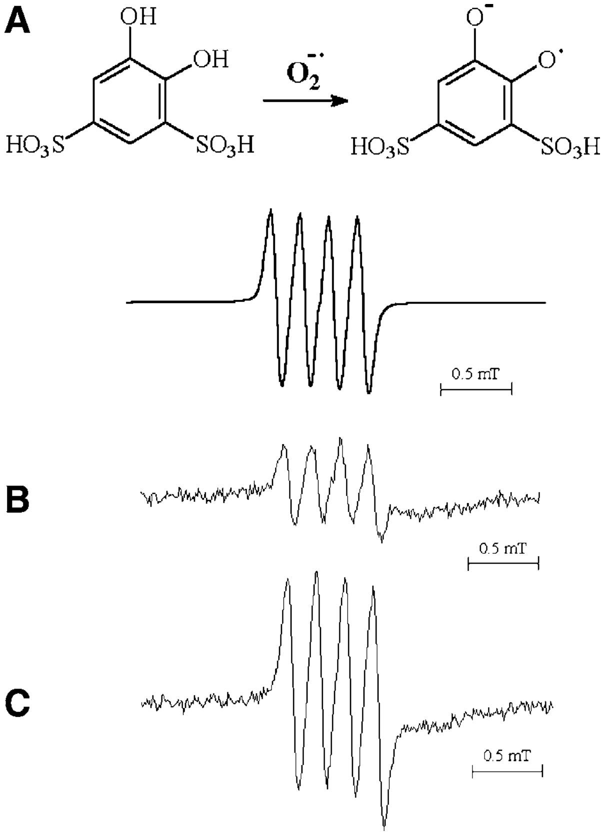

Fig. 5. **(A)** Structure of Tiron and its superoxide radical adduct, Tiron-OOH, EPR spectrum of Tiron-OOH. **(B)** and **(C)**: Spin trapping experiments using Tiron (50 m*M*) in isolated thylakoid membranes (100 μg/mL). Samples were exposed to 200 μ*M* m^{-2} s^{-1} photosynthetically active radiation for 15 min either in the absence **(B)** or in the presence **(C)** of 10 μ*M* methyl-viologen.

active radiation (photoinhibition) (*see* **Note 1**) ***(14–19)***. It is important to note that results of ROS detecting experiments should always be interpreted as part of a complex study integrating as many aspects as possible, such as data on the stress-induced loss of physiological function, oxidative damage of membrane proteins, lipids or DNA, changes in antioxidant capacity, or induction of repair processes, for example.

In the experiment illustrated in **Fig. 4**, isolated thylakoid membranes (*see* **Note 2**) were kept at constant temperature in a temperature controlled glass cuvet. Stress by photoinhibition was achieved by high-intensity (1600 μ*M* m^{-2} s^{-1}) photosynthetically active radiation using a KL-1500 (DMP, Switzerland) lamp.

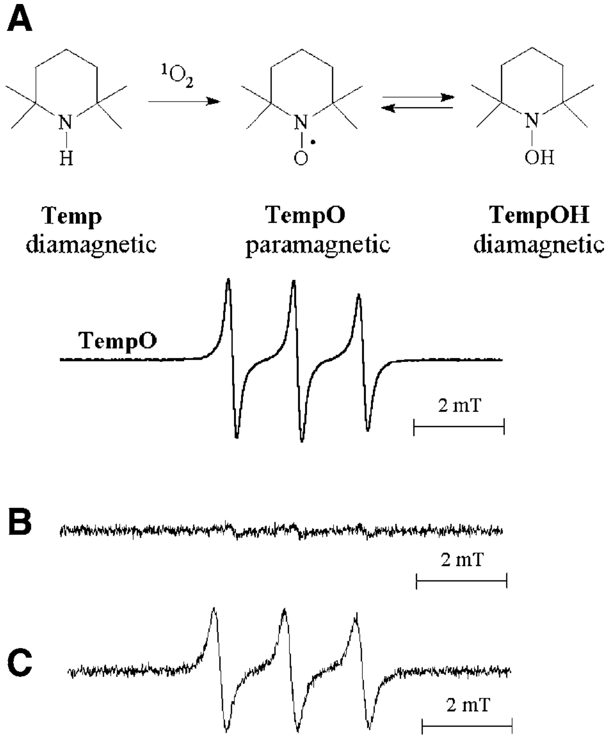

Fig. 6. (**A**) Structures of the sterically hindered amine Temp, its singlet oxygen adduct TempO and hydroxylamine TempOH, and the EPR spectrum of TempO. (**B**) and (**C**): Spin trapping experiments using Temp (10 m*M*) in isolated thylakoid membranes (100 μg/mL). Samples were exposed to 1600 μ*M* m^{-2} s^{-1} photosynthetically active radiation for 15 min, then measured either directly (**B**) or after re-oxidizing ethyl-acetate-extracted TempOH into TempO (**C**).

Samples contained 50 m*M* DMPO (5,5-dimethyl-pyrrolin *N*-oxide) ***(20)***. Because the stability of DMPO adducts in this experimental system was too short for the studied time range of photoinhibition ***(18)***, the spin trap was not kept in the sample through the experiment.

Nitroxide formation from DMPO was measured in short, 5- to 10-min intervals. DMPO was added after various periods of illumination and a sample was withdrawn 5 or 10 min after DMPO addition for EPR measurement. Moving this window of observation through the whole time course of the experiment, it was possible to compare ROS production during the progression of photoinhibition. Typical examples of EPR spectra detected in the early and in the late phase are shown in **Fig. 4**. The latter is a composite spectrum, composed of hydroxyl and carbon-centered radical adducts of DMPO, while the former signal mainly results from the carbon-centered radical adduct ***(14)***.

Although superoxide radicals also form characteristic adducts with DMPO, experiments with this trap can only give a straightforward answer for the involvement of superoxide radicals if these are the main radical species produced ***(21, 22)***. A relatively small concentration of superoxide (manifesting as a small amount of DMPO-OOH as compared to other DMPO adducts) may not be noticed or may easily be detected as DMPO-OH ***(23,24)***. A good alternative is to use Tiron (1,2-dihydroxybenzene-3,5-disulphonate), a spin trap for detecting superoxide radicals ***(25,26)*** (*see* **Note 3**).

Figure 5 illustrates trapping superoxide radicals with Tiron in illuminated thylakoid membranes. Isolated thylakoid membranes (100 μg Chl/mL) were exposed to 200 μ*M* m^{-2} s^{-1} photosynthetically active radiation in the presence of 50 m*M* Tiron for 15 min. Without discussing mechanisms, we only note that only a small portion of superoxide is light stress-related and a similar spectrum was observed using higher light intensities ***(17)***. The addition of 10 μ*M* methylviologen markedly enhances superoxide production, as shown by the increase of the Tiron-OOH adduct (**Fig. 5**).

4. Notes

1. It is important to keep in mind that the added spin traps are in competition for ROS with potential sites of oxidative damage (the natural targets of ROS) and with any antioxidants present in the sample. In this way, failure to observe EPR signal from spin adducts does not necessarily mean that ROS was not produced in the sample. Similarly, when a free-radical adduct is observed, the technique does not tell us whether the trapped ROS is a primary elicitor or a by-product of the studied stress reaction. In addition, all spin trapping techniques underestimate real, *in situ* ROS concentration, because usually only a small fraction of the produced radicals is trapped. This can be somewhat improved by adding more spin trap, but it should be kept in mind that some spin traps inhibit photosynthetic reactions at high concentrations. In our thylakoid membrane experiments, DMPO and Tiron was tolerated up to 80–100 m*M* concentrations, but other spin traps, such as pyridyl-nitrones, were found to be more toxic ***(18)***. Spin adducts (as well as spin traps themselves) may be reduced by plant metabolites into molecules without EPR activity. This can be checked by generating spin adducts chemically (i.e., without the biological sample) and comparing EPR signals in the absence and in the presence of untreated samples. In such experiments, dilution effects created during adding the sample should be taken account. Nitroxides, especially six-membered heterocyclic radicals, such as TempO, the singlet oxygen adduct of Temp (2,2,6,6-tetramethylpiperidine) ***(27)***, are sensitive to reducing agents ***(28)***, which convert the radical to a labile diamagnetic *N*-hydroxylamine (TempOH, **Fig. 6A**). The experiments illustrated in **Fig. 6B,C** were carried out similarly to above ones with DMPO, but using 10 m*M* Temp. When Temp containing samples

of photoinhibited thylakoids were measured directly, there was hardly any TempO signal (**Fig. 6B**). However, when the sample was extracted into an organic solvent, it was possible to restore the nitroxide radical (**Fig. 6C**). To achieve this, photoinhibited samples were mixed with ethylacetate (1/2 V/V) and allowed to separate into two phases for a few minutes. The upper, organic phase (containing the reduced amine, but no photosynthetically active thylakoids) was removed and reoxidized with a catalytic amount (10–30 μg) of PbO_2 before EPR spectroscopy.

2. Thylakoid preparations are good models for studying a number of photosynthetic reactions, but they are less complex than leaves. In addition, many in vivo stress-response reactions occur on different time scales than the possibilities of thylakoid experiments. In this way, a direct extension of spin trapping experiments is not possible, because neither spin traps nor spin adducts endure long enough to report free radical production in leaves during environmental stress experiments lasting for days. Although clearly not a substitute for in vivo monitoring, studies on leaf extracts may prove useful. In these studies, whole plants or detached leaves were exposed to stress conditions as long as necessary, and then frozen in liquid N_2. Frozen leaf powders were ground in a mortar with a phosphate buffer containing the ROS trap, then filtered and measured similarly to thylakoid samples. In these experiments, quick sample processing is essential, so only a small amount of leaves is used. The time between leaf harvesting and EPR measurement should not only be as short as possible, but also uniform for all samples. When evaluating results of these "post-in vivo" experiments it is important to keep in mind that, although samples were collected immediately after the cessation of stress, but as a result of the time delay caused by sample processing, the trapped radicals are not primary products. In this way, the technique is not suitable for qualitative analysis of the ROS produced during the stress, but rather as a characterization of the anti-oxidant–pro-oxidant status of the stressed leaf ***(29,30)***.
3. Direct detection of reactive species with spin trapping is still a dynamically expanding field of biology. Although medical applications are dominant, the technique is gaining more recognition in plant studies. Extensive research is being carried out in several laboratories to develop improved spin traps with higher adduct stability and specificity. A few examples of spin traps, which may prove more versatile than the traps listed in this chapter are: pyridyl- ***(31)***, adamantyl-pyridyl- ***(32)***, glycosylated ***(33)***, or phosphorylated ***(34)*** nitrones. An alternative to EPR spectroscopy is detecting ROS with double sensors, which utilize the principle of spin trapping, but their conversion to nitroxide radical can also be monitored as fluorescence quenching ***(35–37)*** fluorescent sensors.

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24

Stabilization of Photosynthetic Materials

Régis Rouillon, Pierre Euzet, and Robert Carpentier

Summary

Isolated photosynthetic materials have a relatively short active lifetime that limits their effective use. To circumvent this limitation, various immobilization techniques have been designed to improve their stability both under storage and working conditions. The immobilization methods are identified either as chemical or physical methods. In this chapter, two immobilization methods frequently used are described; a physical method based on the entrapment of photosynthetic materials in photo-crosslinkable poly(vinylalcohol) polymer bearing styrylpyridinium groups (PVA-SbQ) and a chemical method where the photosynthetic materials are immobilized by co-reticulation in an albumin-glutaraldehyde crosslinked matrix (BSA-Glu). Different immobilization procedures in relation with various photosynthetic materials are mentioned.

Key Words: Bovine serum albumin (BSA); chloroplast; cyanobacteria; glutaraldehyde; immobilization; photosynthetic; polyvinylalcohol; photosystem (PS) I; photosystem (PS) II; stabilization; thylakoid membrane.

1. Introduction

In thylakoid membranes, light energy is transduced into chemical energy. At this level, light is absorbed by the photosynthetic pigments imbedded in chlorophyll–protein complexes and its energy is transferred to the reaction centers of photosystem (PS) I and II, where a charge separation takes place. During this process, water molecules are cleaved and negatively charged species are formed. These properties confer to thylakoid membranes a great potential for various biotechnological applications. However, the isolated photosynthetic materials have a relatively short active lifetime, which limits their effective use. To circumvent this limitation, various immobilization techniques have been developed to improve the stability of photosynthetic materials both under storage and working conditions. The immobilization methods **(Table 1)** are identified

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Table 1
Main Physical and Chemical Methods Used to Stabilize the Photosynthetic Materials

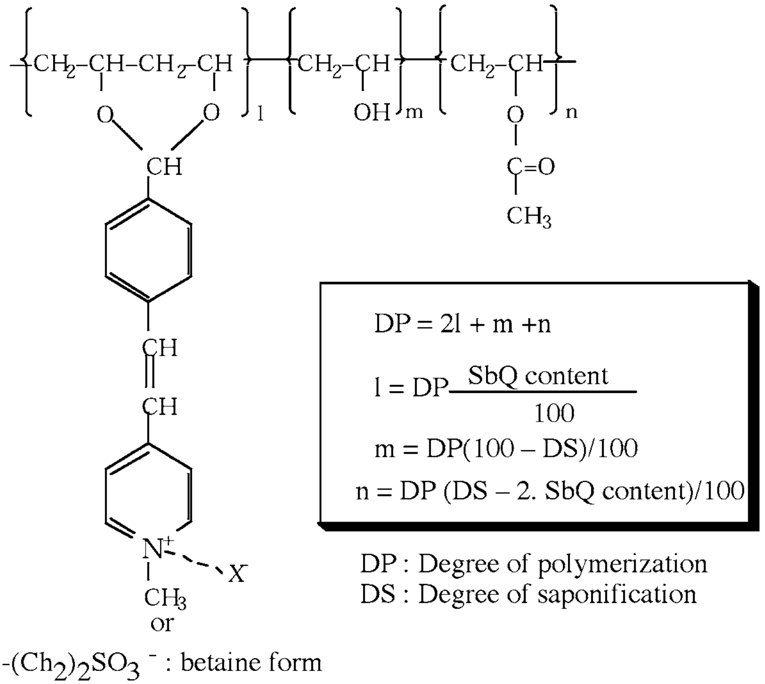
Physical	Chemical
Adsorption	Covalent coupling
• DEAE cellulose	Reticulation
• paper filter disk	• glutaraldehyde
• fiber glass filter disk	Coreticulation: glutaraldehyde
• alumina filter disk	different proteins
Gel entrapment	• gelatin
polysaccharide gel	• collagen
• agar	• bovine serum albumin
• alginate	
• carrageenan	
• agarose	
synthetical gel	
• polyacrylamide	
• polyurethane	
• hydroxyethyl-acrylate	
• poly(vinylalcohol)	
• poly(vinyalcohol) bearing styrylpyridinum groups	

either as chemical or physical methods, depending on whether covalent bonds are established or not ***(1)***.

In this chapter, two immobilization methods frequently used are described; a physical one based on the entrapment of photosynthetic materials in photocrosslinkable poly(vinylalcohol) polymer bearing styrylpyridinium groups (PVA-SbQ) ***(2–7)*** and a chemical one where the photosynthetic materials are immobilized by co-reticulation in an albumin-glutaraldehyde cross-linked matrix (BSA-Glu) ***(6,8–12)***. Different immobilization procedures are mentioned.

In the PVA-SbQ method **(Fig. 1)**, the biological material is stabilized without chemical bonding but is confined into a network established by the polymerized PVA-SbQ. In the BSA-Glu method, the glutaraldehyde builds a network of covalent bondings with the free NH_2 groups of photosynthetic membrane proteins and albumin. The role of albumin is mainly to protect the photosynthetic material against too many bondings so that an adequate biological function is retained.

In comparison to other immobilization methods, the use of PVA-SbQ or BSA-Glu allows a better preservation of the photosynthetic activity. After a



$$DP = 2l + m + n$$

$$l = DP\frac{\text{SbQ content}}{100}$$

$$m = DP(100 - DS)/100$$

$$n = DP\ (DS - 2.\ \text{SbQ content})/100$$

DP : Degree of polymerization
DS : Degree of saponification

Fig. 1. Structure of PVA-SbQ.

storage of 90 d at 4°C in the dark and in dry state, a mixture of chloroplasts and thylakoid membranes entrapped in PVA-SbQ polymers retains 20% of the initial activity (measurements of oxygen production). The same mixture stored at −18°C shows 20% activity after a 427-d storage *(2)*. After immobilization in BSA-Glu, thylakoid membranes maintained in the dark at 4°C remain stable about 200h. After 1000-h storage, the photosynthetic activity was near 40% of initial activity *(8)*.

Various immobilized materials were often used in phytotoxicity tests to detect chemicals that interact with the process of photosynthesis. Despite the largely differing mode of immobilization, the procedures led to strikingly similar detection capabilities for the herbicides tested *(6)*.

2. Materials

2.1. Immobilization Procedures in PVA-SbQ

1. Immobilization buffer: 25 m*M* MOPS-NaOH, pH 7.9, containing 300 m*M* mannitol, 2 m*M* ethylenediamine tetraacetic acid (EDTA), 1% (w/v) bovine serum albumin (BSA) for mixtures of chloroplasts and thylakoid membranes or straight

chloroplasts or thylakoid membranes (*see* **Note 1**); or 20 m*M* MES-NaOH, pH 6.2, for PSII submembrane fractions; or 20 m*M* sodium phosphate solution, pH 7.0, containing 0.15 m*M* NaCl, and 1 m*M* $MgCl_2$, for cyanobacteria.
2. 11% (w/v) poly(vinylalcohol) bearing styrylpyridinium groups solution (PVA-SbQ) (*see* **Notes 2** and **3**).
3. Petri dishes.
4. Microtiter plates.

2.2. Immobilization Procedure in BSA-Glu

2.2.1. Preparation of Samples in Petri Dishes or Test Tubes

1. Immobilization buffer: 50 m*M* phosphate buffer, pH 7.1, for thylakoid membranes; or 20 m*M* Mes-NaOH, pH 6.5, for PSII submembrane fractions; or 20 m*M* Tricine-NaOH, pH 7.8, containing 10 m*M* KCl, 10 m*M* NaCl, and 5 m*M* $MgCl_2$, for PSI submembrane fractions.
2. 20% (w/v) BSA solution.
3. 1.5% (v/v) glutaraldehyde solution.
4. Petri dishes.
5. Test tubes.

2.2.2. Immobilization on the Surface of Screen-Printed Electrodes

1. Immobilization buffer: 15 m*M* MES-NaOH, pH 6.5, containing 0.5 *M* mannitol, 0.1 *M* NaCl, 5 m*M* $MgCl_2$, and 0.05 m*M* chloramphenicol.
2. 10% (w/v) BSA solution in immobilization buffer.
3. 10% (v/v) glutaraldehyde solution.
4. Screen-printed electrodes.

2.2.3. Preparation of Samples in Microtiter Plate Wells

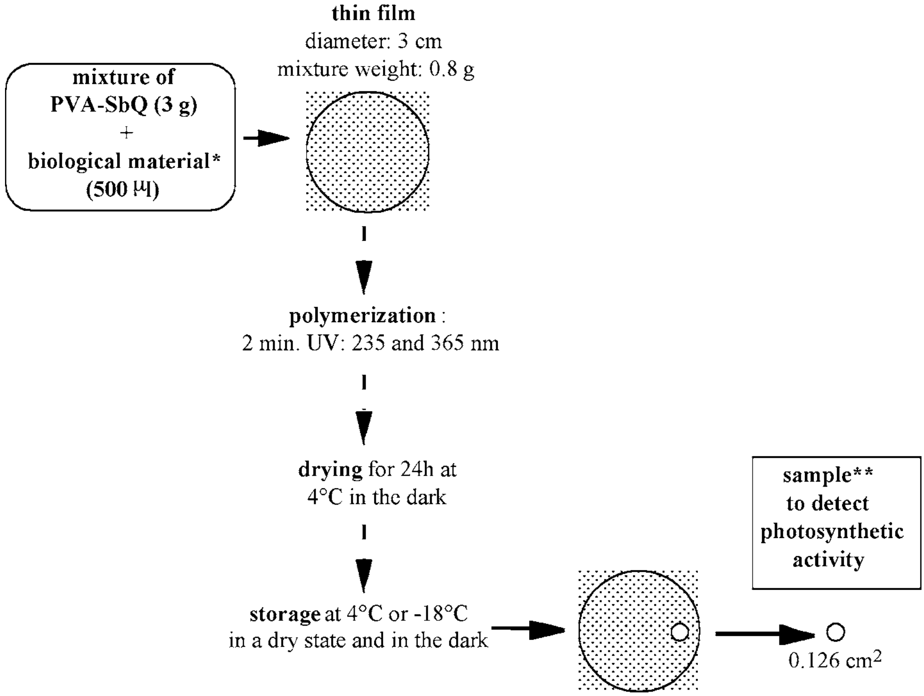
1. Immobilization buffer: 25 m*M* MOPS-NaOH, pH 7.9, containing 300 m*M* mannitol, 2m*M* ethylenediamine tetraacetic acid (EDTA), 1% (w/v) BSA.
2. 20% (w/v) BSA solution.
3. 1.5% (v/v) glutaraldehyde solution.
4. Microtiter plates.

3. Methods

3.1. Immobilization Procedures in PVA-SbQ

3.1.1. Preparation of a Thin Film

This procedure was used to immobilized spinach mixtures of chloroplasts and thylakoid membranes ***(2)***, straight chloroplasts ***(2)***, spinach thylakoid membranes ***(3)***, spinach PSII submembrane fractions ***(4)***, or whole cells of cyanobacterium *Synechococcus* sp. PCC 7942 ***(5)*** (**Fig. 2**).



* Chlorophyll concentration: 3 mg/mL (spinach material) or 0.8 mg/mL (cyanobacteria)

** Chlorophyll content: 6µg (spinach material) or 1.6µg (cyanobacteria)

Fig. 2. Preparation of a thin film with photosynthetic materials immobilized in PVA-SbQ.

1. Weigh 3 g of PVA-SbQ solution.
2. Add 500 mL of photosynthetic material diluted in the immobilization buffer to a chlorophyll concentration of 3 mg/mL for mixtures of chloroplasts and thylakoid membranes *(2)*, straight chloroplasts *(2)*, thylakoid membranes, *(3)*, or PSII submembrane fractions *(4)* and 0.8 mg/mL for whole cells of cyanobacteria *(5)* (*see* **Notes 4** and **5**).
3. Homogenize with a spatula (*see* **Note 6**), and centrifuge the mixture for 5 min at 200*g* (*see* **Note 7**).
4. Spread in a mold to obtain a thin film (diameter: 3 cm. Mixture weigh: 0.8 g), then homogenize the thin film on a mechanical shaker for 5 min at room temperature in the dark.
5. Polymerize under UV light (235 and 365 nm) for 2 min (*see* **Note 8**) and dry for 24 h at 4°C in the dark.

6. Store the thin film at 4°C or –18°C in a dry state in the dark in Petri dishes.
7. Cut samples in the thin film with a hollow punch (*see* **Note 9**).

3.1.2. Preparation of Samples in Petri Dishes

This procedure was used to immobilized spinach thylakoid membranes ***(6)***.

1. Weigh 3 g of PVA-SbQ solution.
2. Add 175 µL of thylakoid membranes preparation (initial concentration of 2 mg chlorophyll/mL) to 375 µL of immobilization buffer.
3. Mix the PVA-SbQ solution and the photosynthetic preparation.
4. Homogenize with a spatula (*see* **Note 6**) and centrifuge the mixture for 5 min at 200*g* (*see* **Note 7**).
5. Deposit the immobilization mixture (60 mg samples) in Petri dishes (*see* **Note 10**).
6. Polymerize under ultraviolet light (235 and 365 nm) for 2 min (*see* **Note 8**) and dry for 24 h at 4°C in the dark.
7. Store the samples at 4°C or –18°C in a dry state in the dark in Petri dishes.

3.1.3. Preparation of Samples in Microtiter Plate Wells

This procedure was used to immobilized spinach thylakoid membranes ***(7)***.

1. Weigh 1.5 g of PVA-SbQ solution.
2. Dilute the PVA-SbQ solution (1/4) with immobilization buffer.
3. Mix with 250 µL of thylakoid membranes preparation (initial concentration of 2 mg chlorophyll/mL).
4. Homogenize with a spatula (*see* **Note 6**) and centrifuge the mixture for 5 min at 200*g* (*see* **Note 7**).
5. Place 150 µL of this mixture in a microtiter plate well and dry the plate during 24 h at 4°C in the dark until 30 µL of solution per well is left.
6. Polymerize the resulting mixture under ultraviolet light (235 and 365 nm) for 2 min (*see* **Note 8**) and incubate at –18°C for 2 d.
7. Store the microtiter plate at 4°C or –18°C in a dry state in the dark (*see* **Note 11**).

3.2. Immobilization Procedure in BSA-Glu

3.2.1. Preparation of Samples in Petri Dishes or Test Tubes

This procedure was used to immobilize spinach thylakoid membranes ***(6,8,9)***, spinach PSII submembrane fractions ***(10)***, or spinach PSI submembrane fractions ***(11)***.

1. Mix 1.65 mL of immobilization buffer specific for the preparation of photosynthetic material used with 1.25 mL of 20% BSA and 1 mL of 1.5% glutaraldehyde (*see* **Note 12**).
2. Incubate for 2 min at room temperature.
3. Add 0.6 mL of photosynthetic material diluted to 3.3 mg chlorophyll/mL ***(8,11)*** or 2 mg chlorophyll/mL ***(6,9,10)*** (*see* **Note 13**) and mix for 3–4 s using a vortex mixer.

4. Distribute the preparation into 80 μL samples in appropriate vessels, such as Petri dishes or test tubes, to obtain the desired shape and volume of immobilized material (*see* **Note 14**).
5. Store for 2 h at –20°C.
6. Thaw the immobilized preparations at 4°C in the dark for at least 2 h before use (*see* **Note 15**).
7. Cover the sample with the resuspension buffer to prevent them from drying.
8. Wash with distilled water before use (*see* **Note 16**).

3.2.2. Immobilization on the Surface of Screen-Printed Electrodes

This procedure was used to immobilized PSII submembrane fractions prepared from the thermophilic cyanobacterium *Synechococcus elongatus* ***(12)***.

1. Mix 970 μL of 10% BSA in immobilization buffer with 970 μL of PSII submembrane fractions diluted to 200 μg chlorophyll/mL.
2. Add 60 μL of 10% glutaraldehyde and mix for 3–4 s using a pipet tip.
3. Place 5 μL on the surface of the working electrode (*see* **Note 17**) and leave the electrode in the dark at 4°C until the mixture solidifies.
4. Use the electrode or store it at –20°C before use.

3.2.3. Preparation of Samples in Microtiter Plate Wells

This procedure was used to immobilized spinach thylakoid membranes *(7)*.

1. Mix 1.25 mL of 20% BSA with 120 μL of 1.5% glutaraldehyde and 1.65 mL of immobilization buffer.
2. Add 200 μL of thylakoid preparation (initial concentration of 2 mg chlorophyll/mL) and mix for 3–4 s using a vortex mixer.
3. Place 50 μL of this mixture in a microtiter plate well and store at –18°C for 2 d.
4. Stored at 4°C before use (*see* **Note 11**).

3.3. Determination of Chlorophyll Concentration

1. Add 10 μL of photosynthetic membranes preparation to 5 mL acetone 80% in a conical tube and mix carefully using a vortex mixer (*see* **Note 18**).
2. Centrifuge in a bench-top centrifuge for a few minutes to remove precipitated proteins.
3. Verify the exact volume (5 mL) and adjust if necessary to compensate the evaporation.
4. Measure the absorbance at 663 and 645 nm.
5. Taking the dilution of the membranes in the acetone solution into account, the chlorophyll concentration (mg/mL) in the membrane preparation is calculated from the following equation:

$$0.5\ [22.22\ (A_{645}) + 9.05\ (A_{663})],$$

in which A_{645} and A_{663} represent the absorbance values at the respective wavelengths.

4. Notes

1. A 1% BSA concentration in the immmobilization buffer increases the stability of the thylakoids membranes *(2)*.
2. The PVA-SbQ polymers were provided by Toyo Gosei Kogyo Co, LTD Japan.
3. Different PVA-SbQ polymers with different degrees of polymerization can be used. With thylakoid membranes, the residual photosynthetic activity and the storage stability increase with the degree of polymerization *(2)*.
4. Chlorophyll concentration was determined according to Arnon *(13)* or Porra and colleagues *(14)*.
5. Additional initial chlorophyll concentrations were also used: 1 or 2 mg/mL.
6. The PVA-SbQ solutions are viscous and cannot be homogenized with a vortex mixer.
7. This operation allows eliminating bubbles. The centifugation speed must be low to avoid sedimentation.
8. An ultraviolet lamp to read chromatographic plates is sufficient.
9. This method allows the evaluation the chlorophyll concentration, e.g., a 0.126 cm^2 sample contains 6 µg of chlorophyll (spinach material) with an initial concentration of 3 mg chlorophyll/mL or 1.6 µg (cyanobacteria) with an initial concentration of 0.8 mg chlorophyll/mL.
10. The immobilized samples used for testing contain 20 µg chlorophyll.
11. It is possible to measure the activity of photosynthetic thylakoid membranes immobilized in microtiter-plate wells using colorimetric methods.
12. Several immobilization buffers were tested for each type of photosynthetic membranes and the buffer used here provides the best retention of native photosynthetic activity and storage stability.
13. The chlorophyll concentration in the immobilization medium is important to obtain the concentration/albumin-glutaraldehyde ratio that provides the best preservation of the photosynthetic membrane integrity together with optimal immobilization.
14. The final immobilized material presents a soft sponge-like green tissue structure. It is important to choose the appropriate vessel for immobilization because it can be used to mold the immobilized material to any shape or volume required.
15. Best activities are obtained after at least 12 h.
16. For some applications, the immobilized material may be crushed into small particles using a mortar and pestle *(10)*.
17. This volume is used for a big electrode printed in PVC. With a smaller ceramic electrode, 0.5 or 1 mL are placed on the surface of the working electrode.
18. The method of Arnon *(13)* was initially used but is now modified according to Porra et al. *(14)*. The sample of photosynthetic membranes should be added to the acetone while mixing. This is necessary to minimize the amount of chlorophyll that remains bound to the precipitated proteins.

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Determination of Phosphoproteins in Higher Plant Thylakoids

Eva-Mari Aro, Anne Rokka, and Alexander V. Vener

Summary

Redox-dependent thylakoid protein phosphorylation regulates both short- and long-term acclimation of the photosynthetic apparatus to changes in environmental conditions. The major thylakoid phosphoproteins belong to photosystem (PS)II (D1, D2, CP43, PsbH) and its light-harvesting antenna (Lhcb1, Lhcb2, CP29) but a number of minor phosphoproteins have also been identified. The detection methods traditionally include the radiolabeling techniques, electrophoretic separation of the phosphorylated and unphosphorylated forms of the protein, and the use of phosphoamino acid antibodies. The recent progress in mass spectrometry (MS) techniques and methods of proteomics allow for successful identification and analyses of protein phosphorylation. In mass spectrometry approaches, exogenous tracer is not needed and natural phosphorylation of proteins can be characterized with high sensitivity yielding the mapping of exact phosphorylation sites in the proteins as well. Various methods for detection of thylakoid phosphoproteins, including the preparation of phosphopeptides for mass spectrometric analyses and techniques for phosphopeptide identification by electrospray ionization MS are described. The experimental protocols for simultaneous identification of multiple phosphopeptides in complex peptide mixtures and for assess of stoichiometry for in vivo phosphorylation of multiple proteins are outlined.

Key Words: CP43; PsbH protein; 12 kDa thylakoid phosphoprotein; light-harvesting protein of photosystem II; mass spectrometry; phospho-threonine antibody; reaction center proteins D1 and D2.

1. Introduction

Several thylakoid proteins undergo reversible phosphorylation, which is regulated by the redox state of thylakoid electron transfer components *(1)*. Numerous thylakoid phosphoproteins have been reported but the most studied ones are associated with photosystem (PS)II. Four of the PSII core proteins, the reaction

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center D1 and D2 proteins, the chlorophyll *a*-binding internal antenna protein CP43, and the small PsbH subunit are well-characterized phosphoproteins ***(2)***. Additionally, the minor antenna protein CP29 (Lhcb4) ***(3)*** and the two major LHCII proteins, Lhcb1 and Lhcb2 ***(2)***, are reversibly phosphorylated. Several novel thylakoid phosphoproteins have recently been reported; the thylakoid soluble phosphoprotein of 9 kDa (TSP9) being of particular interest in showing reversible association with the thylakoid membrane ***(4)***. Although the exact physiological role of thylakoid phosphoproteins is still under discussion, they are likely to have various regulatory functions including the signaling for regulation of gene expression, possibly both in the chloroplast and nuclear compartments. The best characterized thylakoid phosphoproteins become phosphorylated at the threonine residue at or close to the N-terminus ***(5)***. One up to three phosphorylation sites have been mapped in various thylakoid phosphoproteins ***(2,4,6,7)*** Here we describe the most commonly used methods to detect the phosphorylation of higher plant thylakoid proteins: labeling with ^{32}P-adenosine triphosphate (ATP), immunoblotting with phosphothreonine (P-Thr), or protein-specific antibodies and using mass spectrometric approaches. The mass spectrometry (MS) techniques allow the mapping of exact phosphorylation sites in phosphoproteins.

2. Materials

1. P-Thr antibody (Zymed Laboratories Inc., San Francisco, CA and New England BioLabs).
2. AP-Goat Anti-Rabbit IgG (Zymed Laboratories Inc., San Francisco, CA).
3. BSA for blocking (A-7030, Sigma).
4. Polyvinylidene fluoride (PVDF) membrane (Millipore).
5. Whatman 3MM filter paper.
6. A semidry transfer system.
7. TBS: 20 m*M* Tris-HCl, pH 7.5, 500 m*M* NaCl.
8. TTBS: TBS containing 0.05% Tween-20.
9. Chemiluminescence kit (New England BioLabs).
10. Fluorography cassettes or phosphoimager.
11. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
12. Homogenizer (Ultra Turrax T5FU) (IKA-Labortechnik Staufen, Germany).
13. Miracloth (Calbiochem).
14. NaF.
15. Laemmli solubilization buffer and electrophoresis buffer ***(8)***.
16. Porra acetone: 80% acetone buffered with 25 m*M* Hepes-KOH, pH 7.8.
17. [γ-^{32}P] ATP.
18. Sequencing-grade modified trypsin (Promega, Madison, WI).
19. Chelating Sepharose Fast Flow (Pharmacia).
20. $FeCl_3$.

21. $GaCl_3$, caution must be exercised with dissolving $GaCl_3$ in water, as it is an extremely exothermic reaction, which sputters and evolves HCl gas; wear protective clothing and perform in a fume hood.
22. Acetonitrile.
23. ZipTip$_{C18}$ (Millipore).
24. Triflouroacetic acid.
25. Formic acid.
26. Buffer 1: 50 m*M* Hepes-NaOH, pH 7.4, 300 m*M* sucrose, 5 m*M* $MgCl_2$, 1 m*M* Na-EDTA, 10 m*M* NaF and 1% bovine serum albumin (BSA).
27. Buffer 2: 10 m*M* Hepes-NaOH, pH 7.4, 5 m*M* sucrose, 5 m*M* $MgCl_2$ and 10 m*M* NaF.
28. Buffer 3: 10 m*M* Hepes-NaOH, pH 7.4, 100 m*M* sucrose, 5 m*M* NaCl, 10 m*M* $MgCl_2$ and 10 m*M* NaF.
29. Buffer 4: 50 m*M* Na_2HPO_4-NaH_2PO_4 (NaPB), pH 7.5, 0.3 M sucrose and 5 m*M* $MgCl_2$.
30. Osmotic shock medium: 10 m*M* NaPB, pH 7.5, 5 m*M* $MgCl_2$ and 5 m*M* NaCl.
31. Buffer 5: 20 m*M* Tricine, pH 7.8, 5 m*M* $MgCl_2$ and 5 m*M* NaCl.
32. Buffer 6: 50 m*M* Tricine, pH 7.8, 5 m*M* $MgCl_2$ and 0.1 *M* sorbitol.
33. Buffer 7: 330 m*M* sorbitol, 50 m*M* sodium phosphate, pH 7.8, 1 m*M* EDTA, 10 m*M* NaF, 0.15% BSA, 4 m*M* sodium ascorbate and 7 m*M* L-cystein.
34. Buffer 8: 10 m*M* sodium phosphate, pH 7.8, 5 m*M* $MgCl_2$ and 10 m*M* NaF.
35. Buffer 9: 100 m*M* sorbitol, 50 m*M* sodium phosphate, pH 7.8, 5 m*M* $MgCl_2$ and 10 m*M* NaF.

3. Methods

The methods described below outline three possible ways to determine thylakoid protein phosphorylation. The ^{32}P-ATP labeling and immunodetection with P-Thr antibodies are supplementary and allow identification of the phosphoproteins and estimation of their relative amounts. The mass spectrometric analyses provide information on exact phosphorylation sites in the proteins and on the states of phosphorylation for individual proteins. Labeling of proteins with ^{32}P orthophosphate is convenient for unicellular organisms but sometimes cumbersome for intact leaves and therefore the method will not be described here.

3.1. Detection of Thylakoid Phosphoproteins by P-Thr Antibodies

This method has an advantage over labeling experiments in allowing the detection of endogenous level of thylakoid protein phosphorylation in vivo under specific environmental conditions (**Fig. 1**). To avoid dephosphorylation during thylakoid isolation, all the isolation buffers should contain 10 m*M* NaF. When used to study the endogenous phosphorylation levels in vivo, the control experiments should include samples illuminated in far red (<700 nm) and red

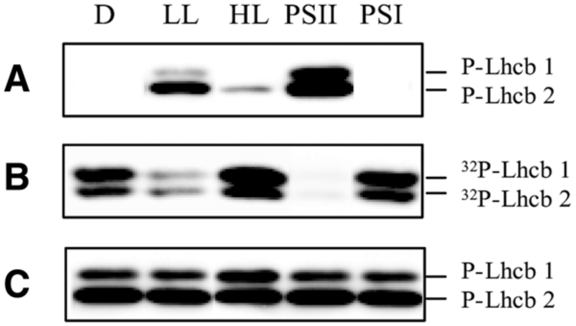

Fig. 1. LHCII protein phosphorylation in differentially light-treated leaves demonstrated by immunoblotting with P-Thr antibodies and by [γ-^{32}P]-ATP labeling. After dark adaptation, the leaves of mature pea plants were illuminated under different light conditions for 2 h before isolation of thylakoid membranes. **(A)** LHCII protein phosphorylation in vivo detected by P-Thr antibody. **(B)** Autoradiogram of thylakoid membranes after in vitro phosphorylation with [γ-^{32}P]-ATP (including 0.4 m*M* cold ATP). **(C)** P-Thr immunoblot following [γ-^{32}P]-ATP labeling of thylakoid proteins, thus demonstrating the total pool of Thr-phosphorylatable LCHII proteins in thylakoid samples. Various light conditions for treatment of intact pea leaves before thylakoid isolation are indicated as D, darkness; LL, 50 μmol photons m^{-2} s^{-1}; HL, 600 μmol photons m^{-2} s^{-1}; photosystem (PS)I and PSII, light favoring PSI and PSII excitation, respectively.

light (650 ± 10 nm) to completely dephosphorylate and phosphorylate the thylakoid proteins, respectively. In addition, a dilution series of thylakoids from leaves illuminated with red light should be included to reveal the linearity of the immunoresponse.

This method is also suitable for studies on regulation of thylakoid protein phosphorylation using intact chloroplasts, isolated thylakoid membranes, or membrane subfractions. The intensity of the immunoresponse with different commercial antibodies differs between various phosphoproteins **(Fig. 2)** (*see* **Note 1**). The origin of immunoresponse with commercial P-Thr antibodies should always be specified ***(9,10)***.

3.1.1. Isolation of Thylakoid Membranes After In Vivo Phosphorylation (a Quick Protocol)

Leaves to be analyzed should be directly frozen in liquid nitrogen. All solutions are supplemented with 10 m*M* NaF to avoid dephosphorylation during thylakoid isolation and subfractionation. BSA (any high-quality grade) and NaF are added before use. Avoid having thiol oxidants or reductants in the isolation buffers. All procedures should be carried out at 4°C under dim light.

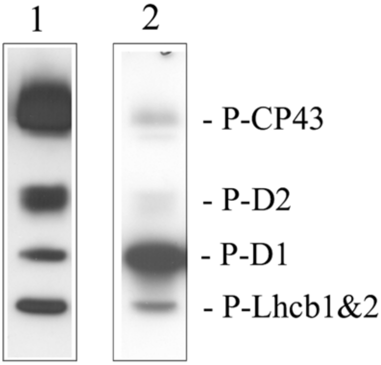

Fig. 2. Immunodetection of thylakoid phosphoproteins by two commercial antibodies. Lane 1, a polyclonal P-Thr antiboby from New England BioLabs and lane 2, a polyclonal P-Thr antiboby from Zymed Laboratiries Inc. Thylakoids were isolated from spinach leaves, which had been illuminated for 30 min under 1500 µmol photons m^{-2} s^{-1} before thylakoid isolation. Thylakoid sample corresponding to 1 µg of chlorophyll was loaded in the well and the proteins were separated as described in **Subheading 3.1.2.**

1. Homogenize leaves (2 g) in 5 mL of a grinding buffer (buffer 1) for 5 s with a homogenizer/ultraturrax.
2. Filter the homogenate through Miracloth and centrifuge at 1500*g* for 4 min.
3. Resuspend the pellet in a lysis buffer (buffer 2) (4 mL) and centrifuge at 3000*g* for 5 min.
4. Finally resuspend the thylakoid pellet in a storage buffer (buffer 3) (50–100 µL).
5. Determined the chlorophyll concentration ***(11)***.
6. Freeze the thylakoids rapidly in liquid nitrogen and store at –70°C (*see* **Note 2**).

3.1.2. Separation of Thylakoid Proteins by SDS-PAGE and Transfer of Proteins to a PVDF Membrane

SDS-PAGE is run according to Laemmli ***(8)***.

1. Prepare 0.75–1.0 mm thick polyacrylamide gels (160 × 200 mm) using 15% acrylamide with 6 *M* urea in the separation gel.
2. Solubilize the thylakoid sample with Laemmli sample buffer for 5 min at 65°C.
3. Load the thylakoid sample corresponding to 1 µg chlorophyll in each well.
4. Run the gels at 10 mA/gel for 16 h (*see* **Note 3**).
5. Transfer the proteins to a PVDF membrane (Immobilon-P) according to manufacturers instructions (*see* **Note 4**).

3.1.3. Visualization of Proteins With P-Thr Antibody

1. Block the membrane with 5% BSA (Sigma A-7030), prepared in TBS, for 1 h.
2. Incubate the membrane in primary antibody overnight (*see* **Note 5**).

3. Wash the membrane four times for 5 min with TTBS.
4. Incubate the membrane in the secondary antibody for 1–2 h.
5. Wash the membrane five times for 5 min with TTBS and two times for 5 min with TBS.
6. Detect phosphoproteins using a chemiluminescence kit according to manufacturers instructions (*see* **Note 6**).
7. For relative quantifications, scan the blots and compare intensities with controls included in the gel side by side with the samples.

3.2. Immunodetection With Protein-Specific Antibodies

For some thylakoid proteins it is also convenient to separate the phosphorylated and nonphosphorylated forms of the protein based on the slightly slower mobility of former during SDS-PAGE and then use a protein-specific antibody to detect both forms of the protein ***(9,12,13)***. For quantitative purposes, care should be taken to verify that both forms of the protein crossreact equally with the antibody. Successful separation of the phosphorylated and nonphosphorylated forms of the PSII D1 protein can be obtained using the following conditions.

3.2.1. Electrophoretic Separation of the Thylakoid Proteins and Visualization of D1 Protein Phosphorylation

1. Prepare and load gels as described in **Subheading 3.1.2.**
2. Run the gels at 30 mA/gel for 16 h (*see* **Note 7**).
3. Transfer the proteins to PVDF membrane according to manufacturer's instructions.
4. Block the membrane with 6% fat-free milk, prepared in TBS, for 1 h.
5. Incubate the membrane in primary, D1 antibody overnight.
6. Continue as described under **Subheading 3.1.3.** from the **step 3** onward.
7. Quantify the immunoresponse using a laser densitometry and a software package for an image quantification. Use appropriate standards and take care to work on the linear response range (*see* **Note 8**).

3.3. Labeling of Thylakoid Phosphoproteins With ^{32}P-ATP

This method is convenient for intact chloroplasts and isolated thylakoid membranes. Because the method detects those phosphoproteins not already phosphorylated in the samples, it is important to isolate chloroplasts/thylakoids from plants kept under conditions that do not favor phosphorylation. If no labeling of phosphoprotein is detected for a particular protein known to be a phosphoprotein, either the pool of phosphoprotein is already phosphorylated in the sample under study or the kinase responsible for phosphorylation is inactive. When using [γ-^{32}P]-ATP labeling it is convenient to check the endogenous phosphorylation level of the protein under interest first with P-Thr antibodies **(Fig. 2)**.

3.3.1. Isolation of Thylakoids for In Vitro Labeling With ^{32}P-ATP

1. Homogenize leaves (20 g) in 40 mL of preparation buffer (buffer 4) three times for 3 s in a rotating-knife blender.
2. Filtrate the homogenate through four layers of nylon net (a pore size of 20 μL) or Miracloth.
3. Centrifuge the filtrate 1000*g* for 3 min.
4. Resuspend the pellet in the preparation buffer (buffer 4) (40 mL) and centrifuge 2400*g* for 5 min.
5. Resuspend the pellet in 40 mL of the osmotic shock medium and homogenize three times with a Potter homogenizer.
6. Centrifuge 2400*g* for 5 min and wash the pellet with a washing buffer (buffer 5).
7. Centrifuge 2400*g* for 5 min and finally resuspend the thylakoid pellet in 3 mL of a phosphorylation buffer (buffer 6).

3.3.2. Labeling of Thylakoid Proteins With [γ-^{32}P]-ATP and Visualization of the Phosphoproteins

1. Incubate thylakoids (0.3 mg chlorophyll/mL) in the presence of 0.1 m*M* [γ-^{32}P]-ATP (0.02 mCi/mg chlorophyll) under a photon flux density of 300 μ*M* photons m^{-2} s^{-1} at room temperature for 30 min (*see* **Note 9**).
2. Spin down the thylakoids in an Eppendorf centrifuge with full speed for 1.5 min and wash the excess label with cold phosphorylation buffer.
3. Centrifuge as above and resuspend the pellet in suitable buffer for further study.
4. Separate thylakoid proteins with SDS-PAGE as described under **Subheading 3.1.2.**
5. Dry the gel between two cellophane sheets and expose to X-ray film at room temperature or use the phosphoimager. Work on the linear response range.

3.4. Mass Spectrometric Analyses of Phosphoproteins

The methods described below outline preparation of the surface-exposed peptides from thylakoid membranes, enrichment of the phosphopeptides by immobilized metal affinity chromatography (IMAC), identification of phosphopeptides using electrospray ionization mass spectrometry (ESI-MS), sequencing of the phosphorylated peptides by tandem MS, and measurement of phosphorylation states for thylakoid proteins using liquid chromatography-mass spectrometry (LC-MS).

3.4.1. Preparation of the Surface-Exposed Peptides From Thylakoid Membranes

For preparation of thylakoid membranes:

1. Homogenize 20 g of Arabidopsis or spinach leaves with a blender in 170 mL of ice-cold preparation buffer (buffer 7).
2. Filtrate the suspension through four layers of Miracloth and centrifuge for 3 min at 1000*g*.

3. Resuspend the chloroplast pellet in 60 mL of the preparation buffer and centrifuge for 5 min at 3000*g*.
4. Resuspend the pellet in 50 mL of lysis buffer (buffer 8) and homogenize five times in a Potter grinder.
5. Centrifuge for 5 min at 7500*g*.
6. Wash the pellet of thylakoid membranes twice with the wash buffer (buffer 9) and three times with 25 m*M* NH_4HCO_3 (pH 8.0), 10 m*M* NaF (*see* **Note 10**). Centrifuge the thylakoids for 5 min at 6000*g* after each resuspension.
7. Finally, resuspend the thylakoid pellet to 3 mg of chlorophyll per mL in 25 m*M* NH_4HCO_3 (to the final volume about 2–3 mL, *see* **Note 11**).

For preparation of the surface-exposed peptides from thylakoid membranes:

1. Incubate the thylakoid suspension with sequencing-grade modified trypsin (Promega) (8 μg trypsin/mg chlorophyll) at 22°C for 90 min (*see* **Note 12**).
2. Freeze, thaw, and centrifuge the digestion products for 20 min at 14,000*g*.

The supernatant containing released thylakoid peptides is used for mass spectrometric analyses directly or after enrichment of the phosphopeptides by immobilized metal affinity chromatography (IMAC).

3.4.2. Enrichment of the Phosphopeptides by IMAC

Enrichment of the phosphopeptides facilitates their subsequent identification and sequencing by MS. Phosphopeptides are affinity enriched from the thylakoid peptides (prepared as described in **Subheading 3.4.1**) by chromatography with immobilized Fe(III) or Ga(III) columns (*see* **Note 13**).

1. Pack columns with 50 μL of Chelating Sepharose Fast Flow (Pharmacia) beads.
2. Wash with 0.3 mL of water, followed by 0.3 mL 0.1% (v/v) acetic acid and charge with 0.3 mL of 0.1 *M* $FeCl_3$ or $GaCl_3$.
3. Wash the unbound salts with 0.5 mL of 0.1% (v/v) acetic acid.
4. Mix the thylakoid peptides (0.2–0.3 mL) with an equal volume of 20% acetic acid and load onto the columns.
5. After washing the columns twice with 0.2 mL of 0.1% (v/v) acetic acid, elute the phosphopeptides with 300 μL of 20 m*M* of nonbuffered Na_2HPO_4.

The phosphopeptides enriched by IMAC could be directly analyzed by HPLC-MS. However, for ESI-MS without prior HPLC they first should be desalted on ZipTips (Millipore).

1. Prewet $ZipTip_{C18}$ with 50% acetonitrile in water and equilibrate with 0.1% triflouroacetic acid in water.
2. Load the peptides by pipeting of 10–20 μL of the peptide sample.
3. Then wash the $ZipTip_{C18}$ with 1% triflouroacetic acid in water.
4. Elute the desalted peptides by 10 μL of 50% acetonitrile in water.

3.4.3. Identification of phosphopeptides Using ESI-MS

For selective identification of phosphopeptides in complex peptide mixtures, the precursor-ion scanning analyses by ESI-MS in negative ion mode is preferable (*see* **Note 14**). This type of mass spectrometric analysis could be performed using electrospray ionization triple quadrupole or hybrid (quadrupole-TOF) mass spectrometers (*see* **Note 15**) equipped with either normal ion spray or nanoelectrospray ion source. The samples after desalting on ZipTips (*see* **Subheading 3.4.2.**) should not be acidified with triflouroacetic acid or formic acid, which decreases negative ionization of the peptides. The peptides released from the surface of thylakoid membrane by trypsin (*see* **Subheading 3.4.1.**) may be subjected to direct analysis being dissolved in water with 25 m*M* NH_4HCO_3.

The nanoelectrospray capillaries are loaded with 2 µL of peptide solution and negative ionization mode full-scan spectra are first recorded to determine the range of mass to charge ratios (m/z) of the major negative molecular ions present in the peptide mixture. Then, the precursor-ion scanning analyses with detection of phosphoryl ions –79 m/z (PO_3^-) and –97 m/z ($H_2PO_4^-$) are performed. The instrument settings from the software for the particular mass spectrometer are used for the full scanning and precursor-ion experiments. The typical result of the precursor-ion scanning experiment for thylakoid peptides is shown in **Fig. 3.** This spectrum was obtained using a hybrid mass spectrometer API Q-STAR *Pulsar i* equipped with a nano-electrospray ion source. Using of the Q-STAR mass spectrometer with the *Pulsar* hardware option has a special advantage for the precursor-ion scanning experiments because it increases the intensity of the phosphoryl ion signals by the order of magnitude.

The precursor-ion scanning experiment provides information on the m/z for phosphorylated peptides present in the mixture. To determine the mass of the corresponding phosphopeptides the full-scan spectrum should be examined in the regions of m/z with the signals in precursor-ion experiment. This allows determination of the charge state of the corresponding phosphopeptide and calculation of its mass. The latter is used for calculation of the m/z for the positive ions of corresponding phosphopeptides that are then subjected for tandem MS as described in **Subheading 3.4.4.**

3.4.4. Sequencing of the Phosphorylated Peptides by Tandem Mass Spectrometry

The electrospray ionization MS used for identification of the phosphorylated peptides (*see* **Subheading 3.4.3.**) are also exploited for fragmentation and sequencing of these peptides. To this end, the peptides should be ionized as positively charged, protonated ions. To increase the positive ionization the peptide solutions (*see* **Subheadings 3.4.1.** and **3.4.2.**) are acidified by addition

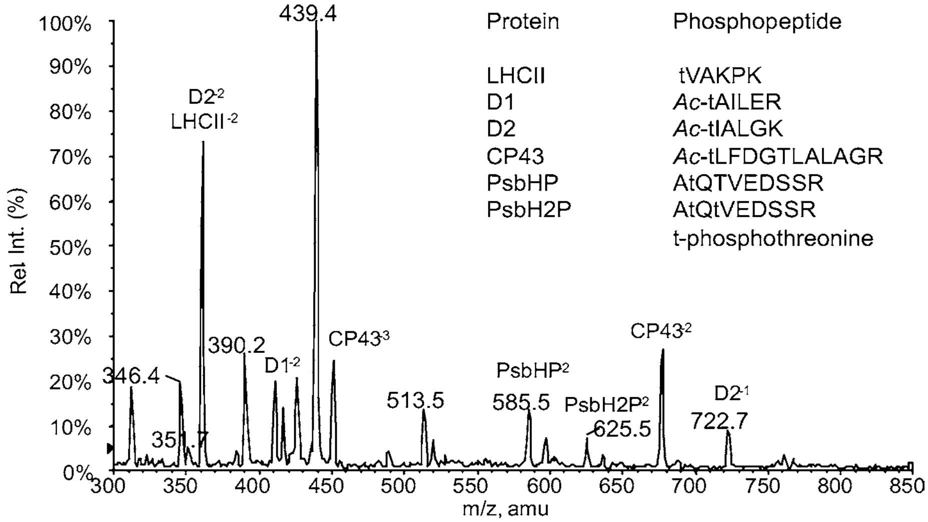

Fig. 3. The precursor of –79 m/z ion (PO_3^-) spectrum for the mixture of the peptides released from the thylakoid membranes of *Arabidopsis thaliana* by trypsin. The spectrum is a result of 100 scans accumulated on a hybrid mass spectrometer API Q-STAR *Pulsar i* (Applied Biosystems, Foster City, CA) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark). The positions of the signals produced by the major phosphopeptides present in the mixture are indicated by the names of corresponding proteins with the superscripts showing the negative charge state of corresponding ions. The sequences of the phosphopeptides are presented in the insert.

of formic acid to the final concentration 1–2% prior to the analyses. Complete sequencing of the phosphorylated peptides requires clean selection of their molecular ions for fragmentation (*see* **Note 16**), which is usually not feasible from the total nonfractionated mixture of thylakoid peptides (*see* **Subheading 3.4.1.**). The mixtures of the phosphopeptides enriched by IMAC (*see* **Subheading 3.4.2.**) are suitable for direct tandem MS and sequencing.

The nanoelectrospray capillaries are loaded with 2 µL of the peptide mixture in 50% acetonitrile in water with 1% formic acid and positive-ionization mode full-scan spectra are first recorded. These spectra are analyzed for the presence of the ions with m/z ratios calculated from the results of the precursor ion analyses (*see* **Subheading 3.4.3.**), according to the mass and possible ionization states of these phosphopeptides. The candidate ions are then selected and subjected to collision-induced dissociation. This is performed using the same sample but the product ion-type of measurements with the instrument settings recommended by vendor of the particular mass spectrometer. Interpretation of the product ion spectra generally allows for determination of the peptide sequence, including even complete *de novo* sequencing. The latter is usually feasible for the peptides not longer than 20 amino acids including phosphopeptides (*see* **Note 17**).

3.4.5. Measurement of Phosphorylation States for Thylakoid Proteins Using LC-MS

When the masses of the phosphorylated peptides present in the peptide mixture released from the thylakoid membranes are determined, the stoichiometry of phosphorylation for various thylakoid proteins can be determined by LC-MS. Approximately 20 to 40 µL of the total peptide mixture obtained from the thylakoid membranes (*see* **Subheading 3.4.1.**) is subjected to LC-MS. The peptides are separated on 5 µm C18 MetaChem 150 × 1.0 mm column at a flow rate 20 µL/min. A gradient of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) is distributed as follow: 0% B for the first 3 min; 0% to 20% B from 3 to 20 min; 20% to 70% B from 20 to 105 min; 70% to 99% B from 105 to 115 min. The online detection with positive ion mode is performed using either triple quadrupole or hybrid mass spectrometer with a standard ionspray source. The instrument settings recommended for a particular mass spectrometer are used.

The phosphorylation states for particular thylakoid proteins are calculated from a computer data file accumulated during the LC-MS run. First, the elution positions for the phosphopeptides with the known m/z are determined. Then, the signals of the corresponding nonphosphorylated forms of these peptides, eluted approx 1 to 2 min earlier, are found. The ratio of the sum of the peak intensities for all ionization states of each phosphopeptide to that of the corresponding nonphosphorylated peptide gives the stoichiometry of phosphorylation for the particular protein. Because both phospho- and nonphospho-forms are detected simultaneously in the same sample, the stoichiometry of phosphorylation can be measured for individual proteins from the membrane sample (*see* **Note 18**).

4. Notes

1. Commercial polyclonal P-Thr antibodies show different crossreaction with different thylakoid phosphoproteins. If you are interested in a specific phosphoprotein it is worth checking several available antibodies for best immunoresponse. With P-Thr antibodies it is not possible to compare the phosphorylation intensities of different thylakoid phosphoproteins with each other.
2. Freezing and storage of thylakoids at –20°C should be avoided, because of irreversible aggregation of proteins.
3. Do not run gels containing urea in cold room (4°C), because urea will precipitate.

4. Instead of using different anode and cathode buffers for protein transfer, they can be substituted with one continuous buffer system (48 m*M* Tris-HCl, 39 m*M* glycine, 0.0375% (w/v) SDS and 20% methanol (v/v).
5. There are occasionally large variations in the immunoresponse between different antibody lots from the same manufacture. When you find the most suitable antibody for your purposes, order enough for the entire set of experiments.
6. Nitrocellulose membranes are not compatible with the chemiluminescense kit, which has been mentioned in the Materials section.
7. Separation of the phosphorylated and nonphosphorylated forms of the D1 protein is obtained only if the proteins are allowed to migrate a long distance in the gel. It is good to use colored protein molecular weigh markers to monitor the migration.
8. It is important to ensure that the used antibody will crossreact equally with both the phophorylated and nonphosphorylated protein forms. The antibody raised against a peptide corresponding to amino acids 234–242 of DE-loop of *Synechocystis* 6803 D1 shows similar reactivity to nonphosphorylated and phosphorylated D1 ***(14)***.
9. To avoid dephosphorylation add 10 m*M* NaF to phosphorylation mixture and to all solutions used after that step. However, omit NaF in case the phosphorylated thylakoids are used to study the dephosphorylation of phosphoproteins.
10. The washes of thylakoid membranes with 25 m*M* ammonium bicarbonate (NH_4 HCO_3) are important for two reasons. First, these washes lead to destacking of thylakoids, which increase the accessibility of trypsin to the surface exposed regions of thylakoid membrane proteins. Second, the resulting supernatant of tryptic peptides in 25 m*M* NH_4HCO_3 is compatible with direct analyses by MS.
11. The final resuspension of thylakoid membranes to a high chlorophyll concentration (about 3 mg of chlorophyll per mL) is important for good proteolytic digestion and for a high concentration of the resultant peptides for analyses.
12. It is important to make the proteolytic treatment at 22 to 25°C but not at 37°C, because a number of PSII phosphoproteins are rapidly dephosphotylated by the heat-shock-activated membrane protein phosphatase at 37°C ***(15)***.
13. Using of IMAC with immobilized Fe(III) ions ***(16)*** as well as Ga(III) ions ***(17)*** is recommended because they could be complementary and neither of them is perfect. Specifically, different sets of thylakoid phosphopeptides could be enriched by either of these chromatographic techniques ***(6)***. The enrichment of the phosphopeptides could be increased by methylation of carboxylate groupes in the peptides prior the IMAC ***(18)***, however, such a chemical modification leads also to a number of side reactions in the major thylakoid phosphopeptides (A.V. Vener, unpublished data).
14. The precursor-ion scanning technique allows for selective determination of the mass to charge ratios (m/z) for phosphorylated peptides that produce the diagnostic phosphoryl ions –79 m/z (PO_3^-) and –97 m/z ($H_2PO_4^-$) ***(4,19,20)***. In this type of mass spectrometric experiment only the phosphorylated peptides present in the peptide mixture are detected, because the detector is "blind" to all other, but the phosphoryl fragment ions, and nonphosphorylated peptides do not produce signals.

15. In case of triple-quadrupole mass spectrometer the time required for a single precursor scan is shorter than in hybrid machine. This allows for accumulation of a greater number of scans during the same experimental time if the triple quadrupole is used. However, hybrid mass spectrometers have higher sensitivity than triplequads. Moreover, both diagnostic phosphoryl ions –79 m/z (PO_3^-) and –97 m/z ($H_2PO_4^-$) could be detected in the same experiment on hybrid machine, although separate experiments should be performed for detection of these ions by triple quadrupole. Detection of both ions is important because the intensity of the –79 m/z and –97 m/z signals differs significantly for different thylakoid phosphopeptides ***(6)***.
16. The ions of individual phosphopeptides should be selected for fragmentation. The deconvolution of the phosphopeptide sequence is extremely hard if two or three different peptide ions penetrate through the selection window and fragment simultaneously. Thus, the width of the selection window could be narrowed to 0.7 atomic units. The narrowing of the selection increases the quality of the fragmentation spectra, but reduces the intensity of the signals. Accordingly, if the parent phosphopeptide ion is not in close vicinity to the other peptide ions the selection window could be broaden to 2 or 3 atomic units width, which would increase the signal intensity due to fragmentation of all isotopic forms for the phosphopeptide.
17. The fragmentation spectra of phosphopeptides allow determination of the phosphorylated residues. A phosphoester bond between a phosphoryl group and a peptide is less stable than a peptide bond, which leads to a prominent neutral loss of phosphoric acid (H_3PO_4, 98 Da) from the positively charged peptide ions ***(6,19, 20)***. The neutral loss of the phosphoric acid also occurs in the fragment ions containing phosphorylated residue. The series of y (C-terminal) and b (N-terminal) fragment ions without the neutral loss (originated from nonphosphorylated fragments of the peptide) together with the distinct ions that underwent the neutral loss (originated from phosphorylated peptide fragments) in each spectrum usually allow identification of the phosphorylation sites.
18. The intensity of ionic species detected by mass spectrometry depends on the nature of a particular peptide and its ionization properties. Consequently, quantitative comparisons of different peptides are generally not allowed. However, because phosphorylation of a peptide adds just 80 m/z (HPO_3) and does not change the ionization state of the peptide under acidic conditions, quantitation of a phosphopeptide relative to its parent peptide is possible using LC-MS in the positive ion mode ***(6)***.

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26

Identifying Photoprotection Mutants in *Arabidopsis thaliana*

Jan Bart Rossel, Abby Cuttriss, and Barry J. Pogson

Summary

Plants have a range of mechanisms to protect against oxidative damage induced by excess light and environmental stress. One of these processes consists of the detoxification of reactive oxygen species by the ascorbate peroxidase (APX) family of enzymes, which convert H_2O_2 into H_2O. Two of the genes encoding APX in Arabidopsis are induced by high light, namely APX1 and APX2. We have applied a genetic approach to understanding the mechanisms of photoprotection, using APX2 as an indicator of oxidative stress. Transgenic plants containing the reporter gene luciferase linked to the APX2 promoter were EMS mutagenized and kindly provided to us by Mullineaux and colleagues ***(1,2)***. We have screened this mutagenized seed to identify mutants with aberrant photoprotection, based on altered luminescence resulting from altered luciferase activity. Here we describe the screen and steps involved in the identification of the mutations in an effort to identify novel photoprotective genes and products.

Key Words: *Arabidopsis*; ascorbate peroxidase; luciferase; mapping; positional cloning; oxidative stress; photoprotection.

1. Introduction

The survival of plants and consequently, most of life on earth, is ultimately dependent on light. Light energy is converted to chemical energy through the process of photosynthesis. However, exposure of a plant to light exceeding that which can be utilized in photochemistry leads to inactivation of photosynthetic functions (photoinhibition) and oxidative stress owing to the production of reactive oxygen species (ROS) such as H_2O_2, superoxide (O_2^-), hydroxyl radicals, and singlet oxygen ($^1O_2$) ***(3)***. ROS are extremely detrimental to plant survival as they can induce the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and cell as a whole ***(4)***. It is therefore of interest to understand the processes a plant utilizes to protect itself

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from excess light and perhaps enhance these processes, leading to plants more resistant to excess light and related stresses.

Under normal growth conditions, plants have evolved several mechanisms to protect themselves against the adverse effect of ROS formed during cellular metabolism *(5)*, including photoprotective compounds such as carotenoids and vitamin E (α-tocopherol). Another line of defense consists of an antioxidant system that provides protection against ROS produced during normal cellular metabolic activity and photosynthesis. This defense mechanism consists of enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione-*S*-transferase (GST) that can dismutate O_2^- radicals and scavenge H_2O_2 *(6)*.

Some members of the APX gene family, APX1 and APX2, are known to be transcriptionally regulated. They are induced by high light, increased H_2O_2 levels, and are regulated by the redox state of the PQ pool. A reduced PQ pool (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone [DBMIB] treatment) increases APX2 expression whereas an oxidized PQ pool [3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCMU treatment] inhibits expression, even under high light *(1)*.

In order to investigate the regulatory pathways involved in the induction of the oxidative stress indicator, APX2, we have designed a screen (**Fig. 1**) to identify potential photoprotective mutants. This involved screening EMS mutagenized transgenic plants for aberrant APX2 expression *(1)*. The screen was based on altered luminescence of a luciferase reporter gene behind the APX2 promoter *(2)*. The method described here has the advantage of being automated and allows consecutive screening for three classes of mutants; plants constitutively expressing APX2, plants with an altered APX2 response after exposure to high light, and plants in which APX2 exhibited an increased expression 15 h after exposure to 1 h of high light.

Other published photoprotection screens target nonphotochemical quenching (NPQ) *(3)*, increased chlorophyll fluorescence *(7)*, aberrant carotenoid accumulation *(8)*, delayed greening *(9)*, and altered APX induction *(2)*.

2. Materials

1. Plants: EMS mutagenized and non-mutagenized *Arabidopsis thaliana* ecotype Columbia transgenic plants containing the luciferase reporter gene APX2 promoter construct.
2. D-Luciferin firefly, potassium salt (synth.) (BIOSYNTH, Staad Switzerland), make 0.1 *M* aliquots and store in –80°C.
3. Plates: Opaque 96-well cell culture plates (Packard, Australia); black is preferable, white can be used.
4. TopCount NXT micro plate scintillation and luminescence counter (Packard, Australia).

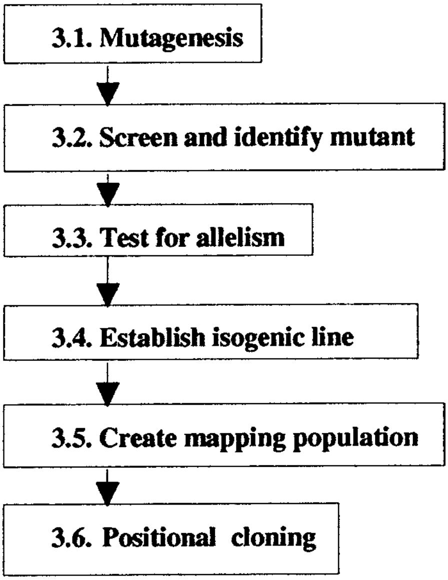


Fig. 1. Mutant identification flow chart.

5. MS medium: MS salts 4.3 g/L, sucrose 0.2% w/v, vitamins 1X, 1% phytagar *(10)*.
6. Agarose 1000 (Invitrogen Life Technologies, cat. no. 10975-035).
7. Molecular markers and standard polymerase chain reaction (PCR) reagents for mapping.
8. DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

3. Methods

3.1. Mutagenesis

3.1.1. Generate or Purchase Mutant Populations

If you wish to saturate the genome with mutations, you need to consider both the number of mutations per seed and the total size of the population. Reports range between 5000 and 125,000 (M1) seeds for EMS mutagenized populations *(11)*, assuming about 100 mutations per seed. M1 populations can be purchased from Lehle Seeds, (www.arabidopsis.com, Round Rock, TX) or generated from existing reporter lines as described by Leyser *(12)* (*see* **Note 1**).

Ecotype selection is important if you anticipate a positional cloning approach to gene identification, as molecular markers are based on sequence differences between ecotypes. Columbia and Landsberg *erecta* ecotypes are preferable because both have been sequenced, giving rise to multiple molecular markers available for this combination *(13)*.

3.2. Screen and Identify Mutants

A crucial step in identifying desired mutants is designing the initial screen. Avoid multiple variables and labor intensive screens. Design the screen to be automated or visual if possible. The screen described here utilizes an EMS mutagenized population of an APX2-luciferase reporter line *(2)*.

3.2.1 Mutant Screen Preparation

1. Sterilize seeds (3 min in 25% bleach, 0.05% Tween-20, 5 min 70% ethanol, wash four times in sterile water) and then plate on MS agar 1% plates and spread out carefully leaving 5 mm space between each seed (*see* **Note 2**).
2. Germinate and grow plants for an average of 4 d (*see* **Note 3**).
3. When emerging roots are approx 5 mm long carefully transfer one plant to an individual well in the 96-well plate using a small spatula. Each well of the 96-well plate contains 200 μL of 0.8% MS agar with 50–100 μ*M* luciferin.
4. Let the plantlets adapt for up to 1 wk so luciferin is evenly taken up throughout the plants (*see* **Note 4**).
5. In each 96-well plate also include one nontransgenic plant and one nonmutagenized APX2-luciferase transgenic plant as controls.

3.2.2 Mutant Screen

1. After 1 wk, measure luminescence generated by luciferase activity using the TopCount micro plate scintillation and luminescence counter (*see* **Note 5**).
2. Measure at 0 h, after 1 h high-light stress and 15 h after return to moderate light. Gain of function mutations could either have increased constitutive (basal) luminescence or a higher (or more rapid) level of expression under high light stress due to altered induction of the APX2-luciferase transgene. Loss of function mutations will either reflect intragenic lesions in the APX2-luciferase transgene or, more interestingly, second site mutations that reduced expression of the transgene.
3. The TopCount plate reader will first need to be normalized with a normalization plate provided by the manufacturer, correcting background and cross-well emission.
4. Depending on the concentration of luciferin, determine how long to count each well (5–20 s) (*see* **Note 6**).
5. Count each plate twice and use average values of each well.
6. Determine an arbitrary cutoff value based on the control luminescence. We chose a cutoff value of a greater than twofold induction or repression of APX2 as compared to nonmutagenized control plants.
7. The same plates are then placed under high light for 1 h.
8. Immediately after the high light exposure, recount each plate using the same parameters as before. This screen will identify loss of function mutants or mutants with extremely high induction of APX2.
9. Plates were then transferred back to the growth cabinet (80 μE, 24 h).
10. The next day, after approx 15 h, the plates were counted again. This screen identified mutants with increased APX2-luc expression as compared to nonmutagenized APX2-*luc* plants.

3.2.3. Rescreening Putative Mutants

Plants selected as a result of the initial screen were potted in soil and allowed to self fertilize. Seeds from these plants were sterilized and plated out as described previously. However, for each putative mutant, 8 plants were plated out in the 96-well plates. The same treatments were carried out as above. If the results were repeatable (*see* **Note 6**), the mutant line was selected for further characterization.

3.3. Allelism

If more than one line displays a similar phenotype, check for allelism. Perform reciprocal crosses ***(14)*** between homozygous mutants and analyze the F1 progeny for the mutant phenotype. Likewise, test for allelism against similar, known mutations (*see* **Note 7**).

3.4. Establish Isogenic Line

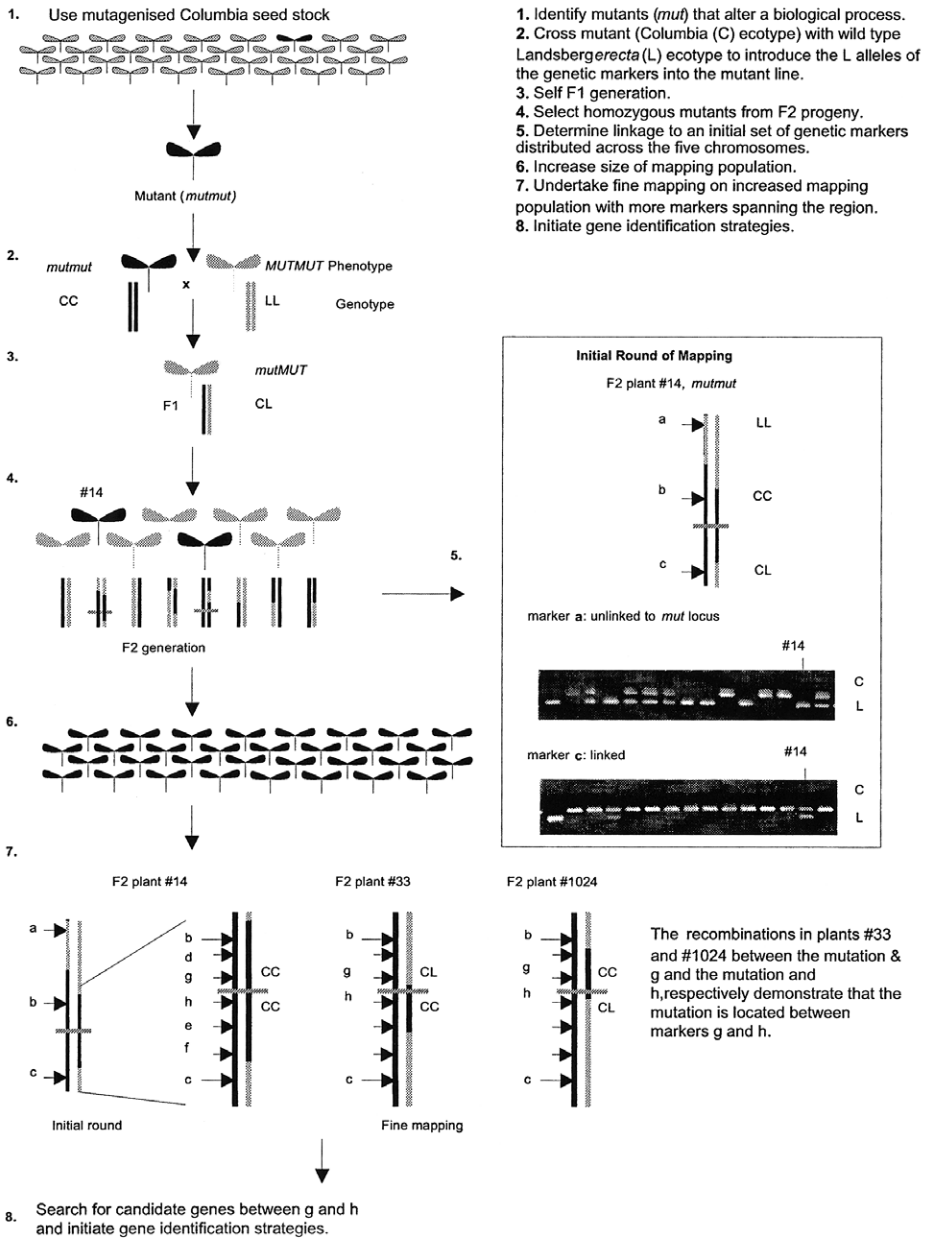
Backcross mutants to the parental ecotype and self the resulting F1 progeny. Repeat backcross three times to "clean up" the mutant line. For EMS mutagenesis there will be numerous mutations per line and each backcross will theoretically exclude half of these spurious mutations in the resulting progeny, which will be homozygous for the altered luminescence phenotype. A segregation analysis during these backcrosses can be informative in the case of a complicated phenotype to determine if the phenotype is pleiotropic, or resulting from other mutations in different genes. A total of four backcrosses will remove about 95% of the superfluous mutations, leaving a near isogenic line. Additionally, the F1 generation can be analyzed to determine the nature of the mutation (e.g., dominant, recessive or semidominant).

3.5. Create Mapping Population

1. Cross the homozygous mutant with alternative ecotype, preferably Landsberg *erecta* wild-type (assuming mutant is in a Columbia background) ***(14)***.
2. Self the resulting F1 progeny to produce a F2 mapping population for use in positional cloning. F2 plants are selected for mapping based on their phenotype, namely those that are homozygous for the mutant phenotype and thus homozygous at that locus.
3. Aim for about 150 mutant plants in the first round of coarse mapping ***(13)*** (*see* **Fig. 2**).

3.6. Positional Cloning

Having identified mutations of interest, the next step is to find the disrupted gene responsible for the altered APX2 photoprotection phenotype.



1. Identify mutants (*mut*) that alter a biological process.
2. Cross mutant (Columbia (C) ecotype) with wild type Landsberg*erecta* (L) ecotype to introduce the L alleles of the genetic markers into the mutant line.
3. Self F1 generation.
4. Select homozygous mutants from F2 progeny.
5. Determine linkage to an initial set of genetic markers distributed across the five chromosomes.
6. Increase size of mapping population.
7. Undertake fine mapping on increased mapping population with more markers spanning the region.
8. Initiate gene identification strategies.

The recombinations in plants #33 and #1024 between the mutation & g and the mutation and h,respectively demonstrate that the mutation is located between markers g and h.

8. Search for candidate genes between g and h and initiate gene identification strategies.

Fig. 2. Identifying genes involved in photoprotection.

3.6.1. Markers

Select markers evenly spread across the genome. A good start is ordering a kit, such as the Arabidopsis MapPairs microsatellite marker set from Research Genetics (cat. no. 20506RG), or designing markers around the Cereon polymorphism database (http://www.arabidopsis.org/Cereon/) and ordering primer sets individually. Alternatively, use the set of markers listed below, compiled by Lukowitz et al. ***(15)*** (*see* **Note 8**) (**Table 1**).

In *Arabidopsis*, the most convenient and commonly used molecular marker types are based on microsatellites and single nucleotide polymorphisms (SNPs). Microsatellite or Insertion/Deletion (In/Del) markers rely on differences between the two ecotypes in short repetitive sequences (also called simple sequence length polymorphisms [SSLPs]) ***(13)***, that can be differentiated by PCR amplification of the microsatellite followed by size fractionation on agarose or acrylamide gels. SNPs can be utilized for molecular marker design in a number of different ways. If the base change between ecotypes results in the loss or gain of a restriction site, then differences can be utilized by way of a cleaved amplified polymorphism marker (CAPs). If a restriction site can be created around a SNP by introducing a mismatch in the primer to create a CAP, then the marker is called derived-cleaved amplified polymorphism (dCAPs). Both of these marker types require PCR amplification of the SNP region, followed by a restriction digest to differentiate between different ecotypes, and are visualized on an agarose gel. There are also other strategies based on denaturing high-performance liquid chromatography and a number of automated high-throughput approaches, which utilize various techniques, including mass spectrometry, microarrays, and DNA sequencing technology.

An advantage of PCR-based markers is that they identify the genotype of both chromosomes. For a particular marker locus, a single “Columbia-size” fragment indicates homozygosity for the Columbia allele, a Landsberg *erecta* fragment homozygosity for the Landsberg allele, or in the case of a heterozygote, both fragments are present. The original mutant was identified in a Columbia ecotype and crossed with a Landsberg *erecta* wild-type plant. Assuming the mutation is recessive, both chromosomes in 100% of F2 plants with the mutant phenotype will be Columbia at the point of the mutation. Near the mutated gene, both chromosomes will be predominantly Columbia alleles for a given marker and hence that locus is “linked”. Further away from the mutation, more recombination events will have taken place, introducing increasingly more Landsberg *erecta* sequence. At positions that are “unlinked” to the mutation, segregation will be random owing to frequency of crossovers during meiosis and thus the chromosomes will average 50/50 Columbia/Landsberg sequence alleles for each locus. This information can be used to determine linkage between markers and the mutant locus; estimate distances between linked markers (*see*

Table 1
Microsatellite Markers (collated by Lukowitz et al., 2000)

Chr.	Position	cM (BAC)	Marker	Forward Primer	Reverse Primer	Product Size (Col/Ler)	MgCl2 (m*M*)
I	10	(F21M12)	F21M12	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG	200/~160	2.0
	39	(T22C5)	ciw 12	AGGTTTTATTGCTTTTCACA	CTTTCAAAAGCACATCACA	128/~115	1.5
	72	(F14J22)	ciw 1	ACATTTTCTCAATCCTTACTC	GAGAGCTTCTTTATTTGTGAT	159/~135	2.0
	81	(F14J16)	nga 280	CTGATCTCACGGACAATAGTGC	GGCTCCATAAAAAGTGCACC	105/85	1.5
	113	(F28P22)	nga 111	CTCCAGTTGGAAGCTAAAGGG	TGTTTTTTAGGACAAATGGCG	128/162	1.5
II	11	(T18C20)	ciw 2	CCCAAAAGTTAATTATACTGT	CCGGGTTAATAATAAATGT	105/~90	2.5
	30	(T26I20)	ciw 3	GAAACTCAATGAAATCCACTT	TGAACTTGTTGTGAGCTTTGA	230/~200	2.5
	50	(F10A12)	nga 1126	CGCTACGCTTTTCGGTAAAG	GCACAGTCCAAGTCACAACC	191/199	2.0
	73	(T7F6)	nga 168	TCGTCTACTGCACTGCCG	GAGGACATGTATAGGAGCCTCG	151/135	2.0
III	20	(MDC16)	nga 162	CATGCAATTTGCATCTGAGG	CTCTGTCACTCTTTTCCTCTGG	107/89	1.0
	43	(MFE16)	ciw 11	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATTC	179/~230	2.5
	70	(F18B3)	ciw 4	GTTCATTAAACTTGCGTGTGT	TACGGTCAGATTGAGTGATTC	190/~215	2.5
	86	(T17J13)	nga 6	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGATC	143/123	1.0
IV	10	(T15B16)	ciw 5	GGTTAAAAATTAGGGTTACGA	AGATTTACGTGGAAGCAAT	164/~144	2.0
	47	(T6G15)	ciw 6	CTCGTAGTGCACTTTCATCA	CACATGGTTAGGGAAACAATA	162/~148	2.0
	65	(F17L22)	ciw 7	AATTTGGAGATTAGCTGGAAT	CCATGTTGATGATAAGCACAA	130/~123	2.0
	104	(T9A14)	nga 1107	GCGAAAAAACAAAAAAAATCCA	CGACGAATCGACAGAATTAGG	150/140	1.5
V	10	(F7P15)	CTR1	CCACTTGTTTCTCTCTCTAG	TATCAACAGAAACGCACCGAG	159/143	2.5
	42	(MQJ16)	ciw 8	TAGTGAAACCTTTCTCAGAT	TTATGTTTTCTTCAATCAGTT	100/~135	2.0
	71	(MIK22)	PHYC	CTCAGAGAATTCCCAGAAAAATCT	AAACTCGAGAGTTTTGTCTAGATC	207/222	2.0
	88	(MFO$_2$0)	ciw 9	CAGACGTATCAAATGACAAATG	GACTACTGCTCAAACTATTCTTCGG	165/~145	1.0
	115	(MSL3)	ciw 10	CCACATTTTCCTTCTTTCATA	CAACATTTAGCAAATCAACTT	140/~130	2.0

Note 9 and **Subheading 3.6.2.)** and hence determine the position of a particular mutation. This is the basis for positional cloning.

3.6.2. Pool Samples and Run Markers

1. Initially, test approximately three molecular markers per chromosome on DNA extracted from the 150 segregating F2 plants. Pooling of samples (preferably in groups of five) for a bulked segregant analysis works well with a large population and considerably reduces cost and time. However, it is not advisable for small pool sizes (*see* **Note 10**).
2. Analyze results by scoring individual plants as CC, CL, or LL for each of the molecular markers where C = Columbia and L = Landsberg (*see* **Note 9**).
3. Determine the recombination frequency as the number of chromosomes with an L allele divided by the total number of chromosomes tested (i.e., two times the number of plants) and multiplied by 100. A chromosome with an L allele for the marker and C for the mutation is a recombinant, one is C for both the marker and the mutant loci has not undergone crossover. A recombination frequency of 50%, which is the maximum that is statisically possible, indicates unlinked markers. Conversely, the lower the frequency of crossovers or recombinations, the closer the marker is to the mutant locus. A recombination frequency of 1% indicates one chromosomal crossover between the mutant locus and the marker's locus per 100 F2 plants and this can be used to calculate genetic distance (centimorgans [cM]) (*see* **Note 9**). Although genetic distance is not directly equivalent to physical distance owing to variable recombination frequencies across chromosomes, a distance of 1 cM is equivalent to about 250 kb in *Arabidopsis*.

3.6.3. Fine Mapping

The initial mapping round and linkage analysis will give some idea of the position of the mutation. Screen more seed from the F2 population for plants that are homozygous for the mutation. Collect both leaf tissue and seed from each plant. The objective of fine mapping is to identify plant(s) with crossovers close to the mutant locus, i.e., <0.1–0.01 cM (that is, a 1/1000 to 1/10,000 event) **(Fig. 2)**. Thus, you need a population in the order of 5000 to be confident of identifying such plants (*see* **Note 11**).

3.6.4. Quick Screen to Identify Recombinant Plants

1. Choose two easy-to-score markers flanking the mutant locus. Prepare "dirty" DNA from leaves collected in **Subheading 3.6.3.** in 96-well format (*see* **Note 12**). You will need to screen the entire population with the two flanking markers within a week as the "dirty" DNA degrades during 4°C storage. This will identify a few hundred recombinants with crossovers (breakpoints) between the markers and mutant locus.
2. Calculate the genetic distance (cM) to determine the approximate location of the mutant locus. Plants that are LL or CL for the marker indicate a recombination event between the marker and the mutant locus, which will be CC.

3. The remaining plants without crossovers, i.e., CC, are uninformative and can be discarded.
4. The recombinant plants with crossovers will help narrow down the region of the mutation and can therefore be used for further fine mapping analysis.
5. Put half an approx 5mm leaf disc into a PCR tube (*see* **Note 13**) (work in 96-well format if your thermocycler is compatible), add 40 µL 0.25 *M* NaOH and grind until pigment starts to go into solution.
6. Heat in PCR block, 100°C for 30 s.
7. Add 40 µL 0.25 *M* HCl and 20 µL Tris-HCl (pH 8.0) 0.25% Triton X-100 and mix gently.
8. Heat in PCR block, 100°C for 2 min.
9. Use 1 µL per 10 µL PCR reaction.

3.6.5. Extract High-Quality DNA

1. Using the Qiagen DNeasy kit, extract DNA from the recombinant plants (**Subheading 3.6.4.**) and proceed with fine mapping.
2. Using the same principle as above, test for recombination between the mutant locus and a range of markers that cover the region. Progressively, your recombinant population will reduce in size as you fine map the mutation.
3. Depending on the distance between a particular crossover event and the mutation, you can resolve the mutant locus down to 10 kb, although this value does vary enormously and the closest crossover event in your recombinant population may be 100s of kb from the mutant locus (*see* **Subheading 3.6.2.**).
4. The next step in gene identification depends on the resolution that could be achieved by fine mapping and the identification of candidate genes.

3.6.6. Candidate Approach

1. To identify candidate genes in the region identified by fine mapping, check sequence databases (*see* **Note 14**) for genes that might account for the phenotype.
2. Amplify candidate genes (standard PCR) from your various mutant alleles and look for polymorphisms such as deletions that are resolvable on a standard gel or sequence the entire gene to identify single base-pair mutations common to EMS mutagenesis (*see* **Note 15**).
3. To check for aberrant transcripts, for example, mutations in the intron/exon splice site, amplify the transcript by RT-PCR and run the products on a standard gel.
4. To identify altered transcript levels use real-time PCR or Northern blot analysis.
5. Finally, to confirm and recreate the mutant phenotype generate knockdown plants for the candidate genes by RNA silencing ***(16)*** or complement the mutation with wild-type candidate genes.

3.6.7. Complementation and Alternative Approaches

If fine mapping doesn't reduce the interval sufficiently and/or it is impossible to pick candidates, narrow down the remaining region by complementing mutant plants with large binary clones ***(17)*** spanning the region (*see* **Note 16**). Additionally, other options to consider include microarrays to probe for differential expression of all genes. It is also worth considering sequencing the whole region, although this is expensive and time consuming.

4. Notes

1. Tagged mutant populations, such as T-DNA insertion lines, are convenient. A stable tag can easily be identified and the interrupted gene identified by TAIL-PCR ***(17)***. Untagged populations, such as chemical, require time-consuming positional-cloning approaches to gene identification. However, chemical mutagenesis can produce a range of subtle mutation types, such as missense mutations, promoter mutations, and knockdowns, for example, which is advantageous in the case of a lethal knockout.
2. Seeds were initially plated out on a higher concentration of MS agar plates than usual as this facilitates transfer to 96-well plates and stops roots from growing deep. Seed clumping can be minimized by resuspending the seed in 0.1% agar (or 0.8% agar at 42°C) prior to plating.
3. It is best to transfer newly germinated seeds to the 96-well plates as the roots of older seedlings make it difficult to transfer and properly place in the agar.
4. Depending on the expression level of the gene of interest it is advisable to determine the ideal concentration of luciferin to be used as this affects the luminescence signal.
5. An alternative approach is to use a cooled CCD camera (–60 to –80°C) in a dark box with image analysis software to visualize induction of luciferase in individual plants *in situ* without transferring to the microtiter plate. This strategy has been successfully applied by a number of researchers ***(18,19)***. However, the quality of the camera (in particular very low dark noise) and dark box is critical as the level of luminescence is very low and may require exposures of 5–20 min.
6. The effect of high light on gene expression may be transient, therefore, do not count too many plates at once. In addition, results from the primary screen can be variable resulting from different plant sizes, altered luciferin uptake, for example. Thus, thoroughly re-screening candidate lines is essential. It would be advisable to assay luciferase activity per gram fresh weight and also check to determine if APX2 mRNA is also correspondingly altered.
7. Several different mutations in the one gene (i.e., alleles) can confirm the disrupted gene responsible for the mutant phenotype. This is particularly important for pleiotropic phenotypes, especially in EMS mutants that can have in the order of 100 mutations per plant.
8. One can run some markers in tandem or duplex to save time (i.e., same PCR tube) provided the product sizes do not overlap and you have appropriate controls. However, some primer sets preferentially amplify larger fragments and can distort the data. PCR conditions; all markers anneal at 55°C, for exact conditions see Lukowitz et al. ***(15)***. The PCR products are analyzed on a 3.5% high-resolution agarose (such as Agarose-1000) gel to resolve the small size differences. When designing markers, this method can easily resolve down to about a 4 base pair (bp) difference between ecotypes, provided the gel is poured hot to reduce "smiling"

and increase resolution. High percentage gels will have to be run significantly longer than a normal 1% agarose gel. Keep marker products short (100 bp or less) and optimize reactions carefully to reduce primer dimer interference.

9. Genetic map distance (*D*) can be determined in terms of map units or centimorgans (cM) using the Kosambi function: $D = 25\ln(100 + 2r/100 - 2r)$, where r = recombination frequency. In fact, for many purposes, recombination frequency (recombinants/parental) is a good approximation of map distance. Recombination frequency (r) is calculated as number of Landsberg alleles for a marker (where homozygous equals 2 Landsberg alleles and a heterozygote equals 1 Landsberg and 1 Columbia) divided by the total number of alleles and expressed as a percentage.
10. If you use significantly less plants in the initial mapping round, a pooled approach may be statistically misleading. Therefore, it is preferable for samples to be kept separate.
11. You will need to screen at least 5000 plants. Some reports have not needed this many, but it is easier to ensure sufficient resolution at this stage, rather than repeat the screen and fine mapping on an additional population later.
12. This is an extremely quick and easy screen and may be easier to do prior to phenotypic scoring of the segregating F2 plants if you have a difficult phenotype to score.
13. Use minimal amounts of tissue, as impurities resulting from too much tissue interfere with the subsequent PCR reactions.
14. The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), MIPS *Arabidopsis thaliana* database (MATDB, http://mips.gsf.de/proj/thal/db/index.html) and the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) are all good resources for annotated sequence data.
15. Several alleles are useful for verification at this stage, especially if you are dealing with EMS mutagenesis, which often yields single base-pair mutations, as mistakes and small variations are common in sequence database.
16. TAC clones are available from the Arabidopsis Biological Resource Centre (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm).

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27

A Simple Method for Chloroplast Transformation in *Chlamydomonas reinhardtii*

Velupillai M. Ramesh, Scott E. Bingham, and Andrew N. Webber

Summary

Photosystem (PS)I is a multi-subunit pigment–protein complex that uses light energy to transfer electrons from plastocyanin to ferredoxin. Application of genetic engineering to photosynthetic reaction center proteins has led to a significant advancement in our understanding of primary electron transfer events and the role of the protein environment in modulating these processes. *Chlamydomonas reinhardtii* provides a system particularly amenable to analyze the structure–function relationship of PSI. *Chlamydomonas reinhardtii* is also a favorable organism for chloroplast transformation because it contains a single chloroplast and grows heterotrophically when supplemented with acetate. *Chlamydomonas* has served as a model organism for the development of chloroplast transformation procedures and the study of photosynthetic mutants generated using this method. Exogenous cloned cpDNA can be introduced into the chloroplast by using this biolistic gene gun method. DNA-coated tungsten or gold particles are bombarded onto cells. Upon its entry into chloroplasts, the transforming DNA is released from the particles and integrated into the chloroplast genome through homologous recombination. The most versatile chloroplast selectable marker is aminoglycoside adenyl transferase (aadA), which can be expressed in the chloroplast to confer resistance to spectinomycin or streptomycin. This chapter describes the procedures for chloroplast transformation.

Key Words: *Chlamydomonas reinhardtii*; chloroplast transformation; electron transfer; photosystem I; and site-directed mutagenesis.

1. Introduction

Photosystem (PS)I is a multi-subunit pigment–protein complex that uses light energy to initiate electron transfer from plastocyanin to ferredoxin. It consists of 13 polypeptide subunits. The two largest subunits, PsaA and PsaB, form a heterodimeric core taht binds approx 100 chlorophyll *a* molecules and the electron transfer cofactors P700, A, A_0, A_1, and F_x ***(1–3)***. Application of genetic

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engineering to photosynthetic reaction center proteins has led to a significant advancement in our understanding of primary electron transfer events and the role of the protein environment in modulating these processes ***(4)***. Much of this work has been restricted to purple bacteria and cyanobacteria in which manipulations of the genes encoding reaction centers is fairly straightforward. In eukaryotes, biolistic transformation of the chloroplast genome has several inherent complications as plants and many green algae are unable to grow in the absence of photosynthesis. The majority of eukaryotes contain several to hundreds of chloroplasts with each organelle containing 80–100 copies of chloroplast DNA. Several of these limitations are addressed by using the green alga *Chlamydomonas reinhardtii*, which contains only a single chloroplast and grows heterotrophically when supplemented with acetate. *Chlamydomonas* has, therefore, served as a model organism for the development of chloroplast transformation procedures and the study of photosynthetic mutants generated using this method ***(2)***. Exogenous cloned cpDNA can be introduced into the chloroplast by using this biolistic gene gun method ***(4,5)***. DNA-coated tungsten or gold particles are bombarded onto cells. Upon its entry into chloroplasts, the transforming DNA is released from the particles and integrated into the chloroplast genome through homologous recombination ***(5–7)***. Because of the efficient homologous chloroplast recombination system, specific deletion or site-directed mutation could be created relatively easily ***(8)***. This chapter describes the procedures for highly efficient chloroplast transformation.

2. Materials

2.1. Growth

1. Strains: *Chlamydomonas reinhardtii* strains CC125 and CC 2696.
2. Medium: Cox *Chlamydomonas* medium (CC) (1x): Tris-HCl base (2.5 g), glacial Acetic acid (1 mL), NH_4NO_3 (0.5 g), $MgSO_4.7H_2O$ (0.1 g), $CaCl_2.2H_2O$ (0.02 g), KH_2PO_4 (0.05 g), KCl (0.1 g), Hutner's trace elements solution (1 mL).
3. Yeast extract should be added to the liquid CC medium (1X) at a final concentration of 0.1% before autoclaving.
4. Spectinomycin.
5. Hutner's trace element solution: For 1 L of trace elements mix, dissolve each compound in the volume of water indicated. The EDTA should be dissolved in boiling water, and the $FeSO_4$ should be prepared last to avoid oxidation.

   EDTA, disodium salt (50 g in 250 mL H_2O) $ZnSO_4$. H_2O (22 g in 100 mL H_2O) H_3BO_3 (11.4 g in 200 mL H_2O), $MnCl_2{\cdot}4H_2O$ (5.06 g in 50 mL H_2O), $CoCl_2{\cdot}6H_2O$ (1.61 g in 50 mL H_2O), $CuSO_4{\cdot}5H_2O$ (1.57 g in 50 mL H_2O), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1.1 g in 50 mL H_2O), $FeSO_4{\cdot}7\ H_2O$ (4.99 g in 50 mL water).

Mix all solutions except EDTA. Bring this mixture to boil and add EDTA. The mixture should turn green. When everything is dissolved, cool to 70°C.

Keeping temperature at 70°C, adjust the pH to 6.7 with 80–90 mL hot KOH (20%). Bring the final solution to 1 L total volume. It should be clear green initially. Stopper the flask with a cotton plug and let it stand for 1–2 wk, shaking it once a day. Filter the solution using two layers of Whatmann no. 1 filter paper. When the solution turns purple, store it in the refrigerator.

2.2. Particle Bombardment

1. 2.5 *M* $CaCl_2$.
2. 0.1 *M* Spermidine.
3. 100% Isopropyl alcohol (high-performance liquid chromatography grade).
4. M10 Tungsten (Bio-Rad, Richmond, CA).
5. Mutated plasmid DNA.
6. Macro carrier (Bio-Rad, Richmond, CA).
7. Biolistic Gun PDS-1000/He (Bio-Rad, Richmond, CA).

2.3. Minipreparation of DNA and Polymerase Chain Reaction (PCR)

1. TEN buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 50 m*M* NaCl.
2. 20 mg/mL Pronase.
3. 10% Sodium dodecyl sulfate (SDS).
4. PCR primers-designed to flank the site of the introduced mutation and to amplify the region of interest.
5. Agarose and agarose gel electrophoresis unit.

3. Methods

The following methods out line the detailed procedure for the chloroplast transformation:

1. Recipient strains and culture conditions.
2. Particle bombardment.
3. Selection and screening for homoplasmic transformants.

3.1. Recipient Strains and Culture Conditions

Chlamydomonas reinhardtii strains wild-type (Wt) CC 125 mt^+ and Wt CC 2696 were obtained from the *Chlamydomonas* culture collection at Duke University and used as recipient strains for site-directed mutation. Strain Wt CC125 contains both PSI and PSII, whereas strain CC 2696 lacks both chlorophyll *a/b* containing light-harvesting complexes and PSII in the thylakoid membrane as it carries the DS521 cab deficiency mutation and a *psbA* deletion mutation. Cells were maintained on 1.5% agar plates containing CC. For chloroplast transformation, a starter culture was initiated by growing the recipient cells heterotrophically in 250 mL flasks containing autoclaved 100 mL CC liquid medium at 25°C under the light intensity of 50 μm photons m^{-2} s^{-1} with constant shaking for 2–3 d according to Harris ***(9)***. Then 1–2 mL of starter culture was transferred to 1 L flasks containing sterilized 500 mL of CC medium and grown to a density of 1–1.5 × 10^6 cells/mL under the same condition as described above. Then the cells were concentrated to 5 × 10^7 cells/mL and 2 × 10^7 cells were spread on plates containing spectinomycin (100 μg/mL) (*see* **Notes 1** and **2**).

3.2. Particle Bombardment

3.2.1. Preparation of M10 Tungsten Particle

1. Weigh 90 mg tungsten particles, then transfer to a disposable glass tube.
2. Keep theRamesh, Bingham, and Webber tube in a beaker and bake in oven at 180°C overnight.
3. Transfer tungsten particles to a 50 mL plastic conical tube and add 15 mL 70% isopropanol and vortex for 3 min.
4. Leave at room temperature for 15 min and collect the tungsten particles by centrifugation at 20,000*g* for 5 min.
5. Wash the pellet three times with 5–10 mL in sterile deionized water followed by centrifugation.
6. Suspend the particles in 1.5 mL sterile 40% glycerol and aliquot 60 μL in sterile microfuge tubes and store at –20°C.

3.2.2. Precipitation of DNA Onto Tungsten Particles

1. Vortex the microfuge tubes containing 60 μL of tungsten particles and add 5 μg of DNA and continue to vortex for 30 s.
2. Add 60 μL of 2.5 *M* $CaCl_2$ while vortexing.
3. Add 12 μL of 0.1 *M* spermidine while vortexing and continue to vortex for 1 min.
4. Leave at room temperature for 15 min.
5. Centrifuge for 10 s and discard the supernatant.
6. Carefully suspend the particles in 250 μL of 100% isopropyl alcohol by repeated pipeting until there is no visible aggregate and vortex for 2 min (*see* **Notes 3** and **4**).
7. Centrifuge for 10 s and discard the supernatant.
8. Resuspend the particles in 60 μL of 100% isopropyl alcohol by repeated pipeting.

3.2.3. Particle Bombardment Using PDS-1000/He

1. Load the 60 μL of DNA precipitated tungsten particles on the thin macrocarrier and allow the particles to air dry.
2. Use a 1350 psi rupture disk.
3. Place the macrocarrier 5/8 of an inch from the rupture disk (*see* **Note 5**).
4. Place the Petri plates containing the recipient cells on the bottom self of the chamber.
5. Evacuate the chamber at least to 28.5 inch Hg and start the bombardment.
6. Incubate the plates under dim light for 2 wk until single colonies appear. Then single colonies are randomly picked and streaked on to new plates and incubated under dim light.

Using the above conditions, transformation efficiencies between 1 × 10^{-5} and 1 × 10^{-4} can be obtained.

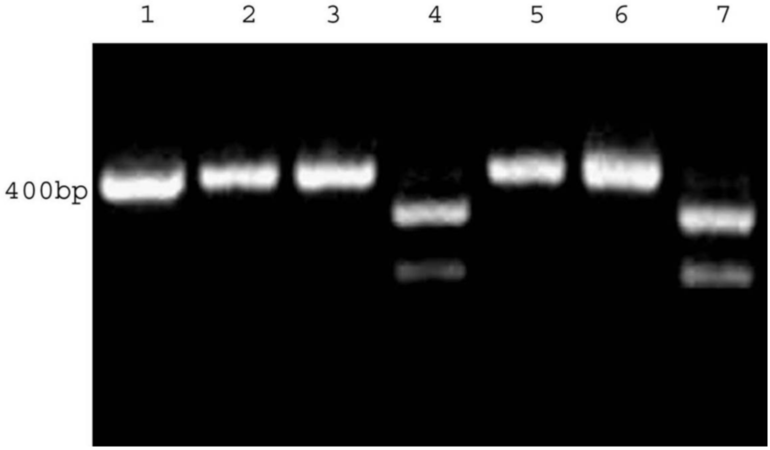

Fig. 1. Analysis of polymerase chain reaction (PCR) product from wild-type and mutant cells. A 400 base pair fragment of PsaB was amplified from total cellular DNA using primers that flank the site of the silent mutation in the donor plasmid that generates a *Eae* I site. The product was either loaded directly onto the 2% agarose gel (lanes 1, 3) or digested with *Eae* I (lanes 2, 4, 5, 6, and 7) before electrophoresis. Complete digestion with *Eae* I (lanes 4 and 7) indicates that all copies of the wild-type *PsaB* gene have been replaced with mutant copy. Wild-type, lanes 1and 2; Mutants containing the *Eae* I site, lanes 3, 4, 5, 6, and 7. Note that *Eae* I did not cut the PCR product from wild-type cells (lane 2).

3.3. Selection and Screening for Homoplasmic Transformants

3.3.1. PCR Analysis

Selection was performed using marker-recycling method as described by Redding et al. ***(10)***. It is important to select for transformant cells in which every copy of cpDNA contains the introduced mutation. PCR can be used to screen for homoplasmy of each colony if the donor plasmid introduces a unique restriction site in the region amplified. Primers are designed to flank the site of the introduced mutation and then used to amplify the region of interest. The PCR product is then digested with the restriction enzyme and size fractionated by 2% agarose gel electrophoresis **(Fig. 1)**. PCR analysis was carried out using the mini-prepared DNA from a patch of plated cells as described below. Single colony selection is repeated for two to three rounds to obtain complete homoplasmic transformants.

3.3.2. Mini-Preparation of DNA From Chlamydomonas Cells for PCR Analysis

1. Transfer a patch of cells (0.5 cm^{-2}) from plate to a microfuge tube containing 400 µL TEN buffer and then suspend the cells by pipeting or vortexing
2. Add 50 µL of pronase and 50 µL of 10% SDS and then mix this solution by inverting two to three times and incubate for 1 h at 50°C.
3. Extract the above solution once with phenol/chloroform/isoamylalcohol (25/24/1) and centrifuge for 5 min.
4. Remove the top layer containing dissolved DNA and precipitate with two volumes of 100% ethanol.
5. Pellet the DNA by centrifugation, wash once with 70% ethanol and then air dry.
6. Dissolve the DNA in 30 µL of sterile deionized water and use 1–2 µL of this DNA for the PCR reaction.

Following the amplification, approx 15–20 µL of the PCR reaction mixture can be digested with restriction enzyme and size fractionated by 2% agarose gel electrophoresis. **Figure 1** shows an example of PCR amplified DNA from wt cells (lane 1, wt; lane 2, wt digested [no introduced enzyme site]) and two mutants (lane 3, undigested mutant; lanes 4, 5, 6 digested) that have a unique *Eae* I site introduced in the transforming plasmid. As shown in **Fig. 1**, the DNA from wt cells does not cut with *Eae* I (**Fig. 1**, lane 2), whereas DNA from the mutants cuts completely (lanes 4 and 6). If the mutants are not homoplasmic then the DNA would not cut completely with *Eae* I. This assay is a very sensitive screening method for homoplasmy. Erickson ***(11)*** reported that PCR can be used to detect one wt copy at a dilution of 1 in 20,000.

4. Notes

1. Several procedures for biolistic transformation of *Chlamydomonas* chloroplasts have been published ***(4,11,12)***. Efficiency of transformation is sensitive to the conditions used. The following three factors; growth stage of recipient cells, preparation of tungsten particles, and conditions of bombardment considerably affect the efficiency of chloroplast transformation. Hence, we have slightly modified the earlier method ***(4)*** especially in the preparation of tungsten particles as described in the methods section. Preparation of tungsten particles by our modified method and dissolving the DNA precipitated tungsten particles in isopropyl alcohol was found to increase the chloroplast efficiency as isopropyl alcohol makes the tungsten particles well separated and prevents from re-aggregation.
2. Growth stage of recipient cells is very important because when cells are grown to a density over 1.5×10^6 cells/mL there is a significant reduction in the transformation efficiency.
3. Quality of DNA is a parameter affecting transformation efficiency. Therefore it is recommended that the CsCl purified DNA ***(13)*** should be used.

4. For resuspending the DNA coated tungsten particles, 100% isopropyl alcohol should be used as it allows the tungsten particles to spread on the macro carrier uniformly without any aggregation.
5. During particle bombardment, the distance between the rupture disk and macrocarrier and the vacuum pressure in the bombardment chamber should be maintained as described in the method.

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28

The Construction of Gene Knockouts in the Cyanobacterium *Synechocystis* sp. PCC 6803

Julian J. Eaton-Rye

Summary

A series of protocols are presented for the storage, growth, transformation, and characterization of wild-type (wt) and mutant strains of *Synechocystis* PCC 6803. These protocols include the isolation of genomic DNA and the strategies required for the construction of specific gene knockouts in this organism. This cyanobacterium has been used widely as a model for photosynthesis research and the sequence of its genome, together with a database of mutants that have already been constructed, is available at CyanoBase (http://kazusa.or.jp/cyano/). The details provided in this chapter do not assume any previous experience in working with cyanobacteria and are intended to enable new investigators to take advantage of a wide-range of gene modification and mutation mapping techniques that have been adapted for use in this system.

Key Words: Cell culture; CyanoBase; DNA isolation; growth curves; mutagenesis; oxygen evolution; *Synechocystis*.

1. Introduction

The cyanobacterium *Synechocystis* PCC 6803 is a naturally transformable Gram-negative prokaryote that performs oxygenic photosynthesis ***(1,2)***. Transformation is achieved via the uptake of DNA by type IV pili and incorporation into the host genome by homologous double recombination ***(3,4)***. A glucose-tolerant strain has been developed that is able to grow photoheterotrophically in the presence of photosystem (PS)II-specific herbicides, such as atrazine or diuron, thereby facilitating the study of PSII through oligonucleotide-directed mutagenesis ***(4,5)***. The widespread use of *Synechocystis* PCC 6803 as a model system led to its selection as the first photoautotrophic organism to have its genome

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sequenced and this information is accessible through CyanoBase at http://www.kazusa.or.jp/cyano/ *(6)*. The substrain selected for genomic sequencing is glucose-tolerant but is noncompetent for transformation with exogenous DNA. A discussion of the different sub-strains together with an overview of the genomic structure and functional genomics of *Synechocystis* PCC 6803 is provided in **ref.** *7*. The objective of this chapter is to describe the basic protocols for growing this organism, constructing gene knockouts, and confirming the genotype and basic phenotype of the resulting mutants. The details presented assume no previous experience working with cyanobacteria but they are intended to provide a series of protocols that may be used for the successful application of more advanced mutagenesis studies with this model organism *(5)*.

2. Materials

2.1. Cell Culture

1. Growth flasks (*see* **Fig. 1**).
2. Cole Palmer Masterflex tubing (CP-96400-25).
3. Millipore 0.2 µm filters (Millex FG50: MISLFG-050-10).
4. Aquarium pump(s).
5. BG-11 stock solutions as shown in **Table 1**.
6. Glucose, herbicide, and antibiotic stock solutions as shown in **Table 2**.
7. Antibiotic-resistance cassettes as shown in **Table 3**. These can be obtained from the author upon request.
8. Sodium thiosulphate.
9. *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES).
10. Bacteriological agar.
11. Disposable 1 mL sterile pipets.
12. Parafilm.
13. Laminar-flow hood.
14. Growth cabinet (*see* **Note 5**).

2.2. The Use of CyanoBase

Software packages for primer design and restriction mapping.

2.3. Cloning Sequence Obtained From CyanoBase

pUC or pBR derivatives as cloning vectors. Specific vectors are suggested in **Subheading 3.3.2.**

2.4. Transformation of Synechocystis *PCC 6803*

1. Sterile test tubes with culture-tube caps.
2. Gloves.
3. 70% Ethanol.

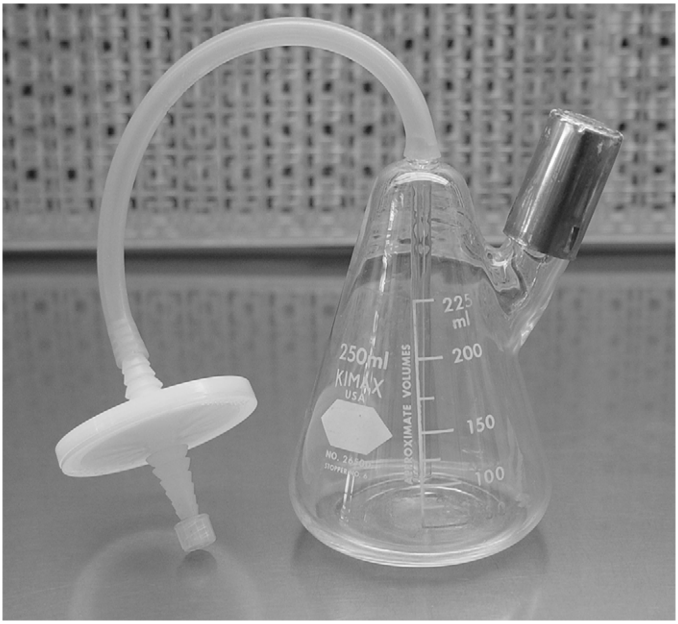

Fig. 1. A modified Erlenmeyer flask used to culture *Synechocystis* PCC 6803 cells based on an original design described by Williams ***(4)***. The 0.2 μm Millipore filter is connected to a glass capillary tube with an outer diameter of 6.0 mm and an inner diameter of 1.7 mm that extends 30 mm above the closed end of the flask and stops 10 mm from the bottom of the flask. The connection is made with Cole Palmer Masterflex tubing. The sidearm is made with glass tubing that has a 20 mm outer diameter and it is covered with a stainless steel culture-tube cap. This whole unit is autoclaved before use.

4. Scissors and small forceps.
5. Nuclepore filters (SN: 145318).
6. Inoculation loop for streaking plates.

2.5. Verification of Mutant Genotype

1. 300-mL Centrifuge bottles.
2. Sodium iodide.
3. Tris buffer 1: 50 m*M* Tris-HCl (pH 8.0), 5 m*M* EDTA and 50 m*M* NaCl.
4. Lysozyme.
5. *N*-Lauryl sarcosine.
6. 45-mL Screw-capped phenol-resistant polypropylene centrifuge tubes.
7. Tris-HCl (pH > 7.8) equilibrated phenol.
8. Chloroform (containing 1/25 isoamylalcohol).
9. 3 *M* sodium acetate (pH 5.0).

Table 1
Composition of Stock Solutions for BG-11 Liquid Medium *(1)*

Stock	Ingredients	Amount
100X BG-11	$NaNO_3$	149.60 g/L
	$MgSO_4 \cdot 7H_2O$	7.49 g/L
	$CaCl_2 \cdot 2H_2O$	3.60 g/L
	citric acid	0.60 g/L
	0.25 *M* NaEDTA, pH 8.0	1.12 mL/L
	Trace minerals	100.00 mL/L
	Deionized water	up to 1 L
Trace minerals	H_3BO_3	2.860 g/L
	$MnCl_2 \cdot {}_4H_2O$	1.810 g/L
	$ZnSO_4 \cdot 7H_2O$	0.222 g/L
	$Na_2MoO_4 \cdot 2H_2O$	0.390 g/L
	$CuSO_4 \cdot 5H_2O$	0.079 g/L
	$Co(NO_3)2 \cdot 6H_2O$	0.0494 g/L
	Deionized water	up to 1 L
1000X	Ammonium iron(III) citrate, brown[a]	0.60 g/100 mL
1000X	Na_2CO_3	2.00 g/100 mL
1000X	K_2HPO_4	3.05 g/100 mL

[a] Also known as ferric ammonium citrate.

10. Absolute ethanol.
11. 70% Ethanol
12. TE buffer: 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA.

2.6. Basic Phenotypic Characterization

1. Clark-type oxygen electrode with FLS1 light source supplied by Hansatech (King's Lyn, Norfolk, UK).
2. Melles Griot OG 515 sharp cut-off yellow glass filter.
3. Neutral density filter set.
4. BG-11 containing 25 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (pH 7.5).
5. Sodium bicarbonate.
6. Methyl viologen.
7. Ascorbic acid.
8. 2,3,5,6-tetramethyl-*p*-phenylenediamine.
9. Potassium ferricyanide.
10. 2,5-dimethyl-*p*-benzoquinone.

Table 2
Additions for BG-11 Liquid Medium

Solution	Stock	Final concentration	Notes
Glucose	1 *M*	5 m*M*	autoclave; store at 4°C
Atrazine[a]	20 m*M*	20 μ*M*	make in methanol; store at –20°C
Diuron[b]	20 m*M*	20 μ*M*	make in ethanol; store at –20°C
Chloramphenicol	50 mg/mL	25 μg/mL	make in ethanol; store at –20°C
Erythromycin	25 mg/mL	25 μg/mL	make in ethanol; store at –20°C
Kanamycin	50 mg/mL	25 μg/mL	filter-sterilize; store at –20°C or 4°C
Spectinomycin	50 mg/mL	25 μg/mL	filter-sterilize; store at –20°C or 4°C

[a] 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine.
[b] 3,4-dichloro-1,1-dimethyl urea (DCMU).

Table 3
Antibiotic-Resistance Markers

Resistance marker	Plasmid from which cassette was derived	Accession or reference
Chloramphenicol	pBR325	L08855
Erythromycin	pRL425	Reference ***(21)***
Kanamycin	pUC4K (Tn 903)	X06404
Spectinomycin	pHP45Ω	K02163

3. Methods

This section will outline:

1. The culturing of *Synechocystis* PCC 6803 cells;
2. The use of CyanoBase to obtain sequence information for the design of PCR primers and cloning strategies;
3. The construction of plasmids for the introduction of mutations into *Synechocystis* PCC 6803 cells;
4. Transformation of *Synechocystis* PCC 6803;
5. The verification of the cyanobacterial mutants' genotype;
6. Measurements of growth curves and oxygen evolution in whole cells;
7. The registration of mutants with CyanoMutants, and
8. Information on additional methods to expand the utility of this system.

3.1. Cell Culture

The wild type (wt) can be obtained directly from the Pasteur Culture Collection (*see* **Note 1**) and the glucose-tolerant strain, utilized in PSII studies, is available on request from the author. The cells are maintained on BG-11 plates at 30°C (*see* **Note 2**).

1. The stock solutions required to make BG-11 medium are given in **Table 1**.
2. To obtain 1 L of BG 11, combine 987 mL of deionized H_2O with 10 mL of 100X BG 11 and 1 mL each of 1000X ammonium iron (III) citrate brown, 1000X Na_2CO_3 and 1000X K_2HPO_4; however, for plates also add 15.0 g/L agar, 10 mL of 10 m*M* TES-NaOH (pH 8.2), and 3 g/L sodium thiosulphate (*see* **Note 3**).
3. After autoclaving, allow the medium to cool to 55°C before adding glucose, herbicides, and/or antibiotics if these are required.
4. The plates should be poured and dried in a laminar-flow hood and cyanobacterial cells should be spread using standard microbiological sterile techniques (*see* **Note 4**).
5. The plates should be placed in a growth chamber under constant illumination at 15–25 $\mu E\ m^{-2}\ s^{-1}$ (*see* **Note 5**).
6. To avoid condensation, the plates should be placed right-side up and to minimize contamination and dehydration they can be wrapped with parafilm.

For successful liquid cultures modified Erlenmeyer flasks are recommended as shown in **Fig. 1**. Modified 200 and 500 mL flasks are generally the most useful but 2 L flasks work well when large cultures are required.

1. Perform inoculation and sampling of flasks in a laminar-flow hood taking all possible care to maintain sterile conditions.
2. Scrape cells from a BG-11 plate using a disposable 1 mL pipet and introduce into the culture flask so that several clumps of cells are clearly visible.
3. Liquid cultures are bubbled using an aquarium pump to pass air through a Millipore 0.2 µm filter; however, before turning on the pump cultures should be left to stand for at least 2 h (an overnight wait is also suitable). This procedure facilitates the growth of the cultures.
4. Cell growth is monitored by measuring the turbidity, or optical density (OD), of the cultures at 730 nm.
5. Liquid cultures should not be grown beyond an $OD_{730\ nm}$ of 2.0 if the culture is to be diluted for further use. To avoid inaccuracy when determining the $OD_{730\ nm}$ of a culture it should be diluted to an $OD_{730\ nm} < 0.4$ to prevent errors resulting from light scattering (NB. an $OD_{730\ nm}$ of $0.25 = 1 \times 10^8$ cells/mL) *(4)*.
6. When glucose, herbicides, or antibiotics are required in liquid media and plates they should be added at the final concentrations supplied in **Table 2**. The antibiotic-resistance cassettes providing resistance to the antibiotics in **Table 2** are derived from the plasmids given in **Table 3**.
7. The wt and mutant cultures can be stored at −80°C in sterile BG-11 containing 15% glycerol.

3.2. The Use of CyanoBase

The initial step in the construction of a gene knockout is to obtain the sequence of the target gene from CyanoBase for the design of PCR primers and the construction of a restriction map of the intended PCR product.

3.2.1. Obtaining Sequence From CyanoBase

1. In an internet browser enter http://www.kazusa.or.jp/cyano/.
2. In CyanoBase select the *Synechocystis* PCC 6803 genome and type the name of the gene you wish to knockout into the box and press *Search*. If you do not know the name of your gene perform a BLAST search (*see* **Note 6**).
4. CyanoBase will provide the unique identifier for this gene in the genome and by clicking on this number a detailed summary of the information available on this gene will be displayed. The nomenclature used to identify specific genes is explained in the notes (*see* **Note 7**).
5. To ensure that adequate sequence is obtained for efficient homologous recombination select 1 kb up-stream and down-stream of the gene by entering the values into the dialogue boxes and press *Submit* (*see* **Note 8**).
6. Save the nucleotide sequence obtained in a format that will enable the sequence to be entered into a standard package that will give a restriction map.

3.3. Cloning Sequence Obtained From CyanoBase

In this section the design of PCR primers and the selection of a suitable vector for cloning the sequence from CyanoBase will be explained.

3.3.1. Designing PCR Primers

Ideally the PCR product should be between 2.0 and 2.5 kb to facilitate cloning. Primers can be designed for the sequence obtained from CyanoBase by using a program for primer design or alternatively primers can be designed by eye. Typically primers should be approx 20 nucleotides long and the 3' end should contain two or three G or C bases. All standard criteria for designing primers apply; such as avoiding sequences that can pair within a primer, primer dimers, and maintaining equal G/C content in both primers of a pair. However, before the primers are designed the restriction map of the sequence should be examined to identify a suitable site within the open-reading frame (ORF) for insertion of an antibiotic-resistance cassette for gene interruption and subsequent selection of the cyanobacterial transformant (*see* **Note 9**). Primers may also be designed across restriction sites if this will simplify cloning or restriction sites can be introduced into the primer, although in the latter case longer primers may be necessary.

3.3.2. Vector Selection

Synechocystis PCC 6803 can be easily transformed with pT7Blue-T (Novagen, Madison, WI) *pUC 19* (New England BioLabs, Beverly, MA), pBluescript (Stratagene, La Jolla, CA) and the pGEM-T (Promega, Madison, WI) vector systems. If the required restriction site for cloning the antibiotic-resistance marker in the ORF is also present in the polylinker of the plasmid this can be initially removed by cutting and blunting the site, followed by religation of the plasmid, before cloning in the PCR insert. It is also important to ascertain if any of the flanking DNA that will be used for recombination contains any sequence for neighboring genes and an appropriate PCR enzyme, with or without proofreading, should be chosen. In the simplest cases, the use of T/A cloning into pGEM-T or pGEM-T Easy is recommended.

3.4. Transformation of Synechocystis *PCC 6803*

Once the gene of interest has been cloned and interrupted the knockout is created by transformation into *Synechocystis* PCC 6803 using the protocol outlined in this section.

3.4.1. Cyanobacterial Transformation

For this procedure ensure that all work surfaces and equipment are sterile (*see* **Note 10**).

1. Start a fresh cyanobacterial culture in BG-11, adding glucose and antibiotics as appropriate. Grow for 2–3 d but do not let the $OD_{730\ nm}$ increase above 0.5 as the transformation efficiency declines in late log phase.
2. In a laminar-flow hood, transfer the cells from the flask to 50 mL sterile Falcon tubes and spin down cells at approx 2760*g* for 5 min at room temperature.
3. Discard the supernatant and resuspend the pellet in 2 mL BG-11 medium and measure the $OD_{730\ nm}$ (*see* **Note 11**).
4. Each transformation will be performed using a 0.5 mL volume of the cells at an $OD_{730\ nm}$ of 2.5. Dilute sufficient cells to an $OD_{730\ nm}$ of 2.5 using BG-11 medium (*see* **Note 12**).
5. Aliquot 0.5 mL volumes of cells (at an $OD_{730\ nm} = 2.5$) into sterile test tubes with culture-tube caps.
6. Add 2–10 μg of DNA in 2–10 μL of TE buffer to each tube and shake. However, leave one tube without the addition of DNA as a control.
7. Place tubes in a growth chamber at 30°C for 6 h in the light and shake the tubes once at 3 h.
8. Prepare Nuclepore filters by cutting into halves or quarters and place onto BG-11 plates in a laminar-flow hood.
9. Place the sterile filter sections (referred to hereafter as filters) onto BG-11 plates that do not contain antibiotics in a laminar-flow hood and plate 200 μL of the cells onto the filters. Allow filters to dry before transferring plates to the growth room (*see* **Note 13**).
10. Grow for 12 h and then transfer the filters to a BG-11 plate that contains the appropriate antibiotic at half the concentration listed in **Table 2**. After 3 d transfer the filters onto BG-11 plates containing the appropriate antibiotic at the concentration given in **Table 2** (*see* **Note 13**).

11. Colonies should appear after 1 or 2 wk. Pick colonies and streak a single colony on a BG-11 plate containing the appropriate additions (e.g., antibiotics) as shown in **Table 2** (*see* **Notes 13** and **14**).
12. To ensure segregation of the transformants restreaks should be performed at approximately weekly intervals for 3 or 4 wk. PSII-specific mutants are normally fully segregated after this time but other mutations may require additional restreaking for several months. It should be noted that a failure to segregate implies that an essential gene has been targeted for disruption.

3.5. Verification of Mutant Genotype

Because *Synechocystis* PCC 6803 cells contain approx 10 copies of their genome it is essential to confirm that all copies contain the mutation. The most rapid method for establishing the complete segregation of gene knockouts is by colony PCR. However, this method may not always work and PCR from isolated genomic DNA may be preferred (*see* **Note 15**). Alternatively, a Southern blot may be performed on the isolated genomic DNA. In this section, the methods for colony PCR screening and isolating genomic DNA are presented (*see* **Note 16**). For Southern blotting the large scale genomic DNA isolation procedure is recommended.

3.5.1. Colony PCR

1. Make up a 50 µL PCR mix in a microfuge tube but do not add the polymerase enzyme at this step.
2. Using an inoculation loop add a cyanobacterial colony to the PCR mix and disperse with low-speed vortexing.
3. Collect the sample with a very short (<2 s) spin in a microfuge.
4. Add the polymerase enzyme and gently mix the sample with the pipet tip. Run the reaction.
5. After the PCR amplification spin for 30 s at maximum speed in a microfuge (typically approx 13,600*g*). Next transfer the supernatant to a clean microfuge tube and analyze the products on an agarose gel.

3.5.2. Rapid Small-Scale Genomic DNA Isolation From Plate Cultures

1. Scrape all the cells from a 3-wk-old plate and resuspend in 200 µL of saturated NaI (2 g/mL) in a microfuge tube and incubate at 37°C for 10 min.
2. Add 1mL distilled, de-ionized (dd)H_2O to dilute the NaI and centrifuge at 13,600*g* for 10 min.
3. Discard the supernatant and resuspend the pellet by pipeting with a 1 mL pipet in 400 µL of Tris buffer 1.
4. Add 100 µL of a 50 mg/mL lysozyme solution and incubate for 10 min at 37°C followed by the addition of 100 µL of a 10% *N*-lauryl sarcosine solution.
5. Gently mix the tube by inversion and incubate at 37°C for 10 min.

6. Add 600 μL of Tris-HCl (pH >7.8) equilibrated phenol and mix gently on a rotating wheel for 20 min.
7. Centrifuge at 13,600*g* for 10 min and transfer the upper aqueous phase to a new tube using a 1 mL pipet with the end removed to avoid shearing the DNA.
8. To this new tube add 600 μL of chloroform (containing a 1/25 volume of isoamylalcohol) and gently mix on a rotating wheel for 20 min.
9. Transfer the upper aqueous phase, as in **step 7**, to a clean tube and ethanol precipitate the DNA by adding a one-tenth volume of 3 *M* sodium acetate (pH 5.0) and two and a half volumes of absolute ethanol. Place the tube at –20°C for 3 h and then collect the DNA by centrifugation at 13,600*g* for 20 min at 4°C.
10. Discard the supernatant and wash the pellet in chilled 70% ethanol (–20°C) to remove salt. Centrifuge for 5 min and aspirate the supernatant. Dry the pellet at room temperature and then resuspend in 50 μL of TE buffer and store at –20°C.

3.5.3. Large-Scale Genomic DNA Extraction From Liquid Culture

1. Weigh a 300 mL centrifuge bottle, add 75–250 mL of cell culture, and centrifuge at 7000*g* for 5 min. Remove the supernatant and weigh the bottle again. Determine the weight of the cell paste (*see* **Note 17**).
2. Resuspend the cell paste in saturated NaI: for 1 g of paste use 2 mL of saturated NaI (2 g NaI per mL H_2O) and incubate at 37°C for 20 min.
3. Fill up the centrifuge bottle with water to dilute the NaI.
4. Centrifuge for 10 min at 2760*g* at 4°C and then pour off the supernatant.
5. Resuspend the pellet in 8 mL of Tris buffer 1, per gram of cells. Resuspend completely by passing through an 18-gage needle.
6. Per gram of cells, add 1.25 mL of a 50 mg/mL solution of lysozyme and mix gently. Incubate at 37°C for 20 min (*see* **Note 18**).
7. Add 1 mL of 10% *N*-lauryl sarcosine (per gram cells), invert tube gently to mix, and then incubate at 37°C for 20 min.
8. Put lysate into a 45 mL phenol-resistant, screw-capped plastic tube and add an equal volume of Tris-HCl (pH >7.8) equilibrated phenol and place parafilm around the lid to prevent leakage (*see* **Note 19**).
9. Mix gently on a wheel for 60 min and then centrifuge at 2760*g* for 10 min at 4°C.
10. Use a pipet with a 5 mL tip (to prevent shearing of the DNA) to remove the aqueous (upper) phase, which should be pink, and place in a clean phenol-resistant tube.
11. Add an equal volume of chloroform (containing 1/25 volume of isoamylalcohol) to the aqueous phase, put parafilm around the lid and mix gently on a wheel for 45 min.
12. Centrifuge at 2760*g* for 10 min at 4°C and transfer the top aqueous phase to another clean phenol-resistant centrifuge tube. For high-quality DNA it is recommended to repeat **steps 8–12** before proceeding to **step 13**.
13. Ethanol precipitate the DNA by adding a one tenth volume of 3 *M* sodium acetate (pH 5.0) and two and a half volumes of absolute ethanol. Shake gently and place tube at –20°C overnight.

14. Centrifuge at 23,000*g* for 15 min at 4°C and then aspirate the ethanol.
15. Wash the pellet with chilled 70% ethanol (–20°C), centrifuge at 2760*g* for 5 min at 4°C and aspirate the ethanol.
16. Put parafilm over the top of the tube and puncture several times with a pin. Dry in a Speedvac (Savant, Holbrook, NY) or equivalent instrument.
17. Resuspend the pellet in 300 µL of TE buffer and store at –20°C in a microfuge tube.

3.6. Basic Phenotypic Characterization

Although the appropriate phenotypic characterization will depend on the specific mutations that have been introduced, growth curves, and oxygen evolution assays provide a simple assessment of photosynthetic performance in mutant strains. Both of these measurements are described in this section.

3.6.1. Photoautotrophic Growth Curves

1. For each strain, grow a starter culture to an $OD_{730\ nm}$ of approx 0.8. These cultures may appropriately contain glucose and antibiotics (*see* **Note 20**).
2. Working in a laminar-flow hood pour 40 mL of the starter culture into a 50 mL sterile Falcon tube and spin-down the cells at 2760*g* for 5 min at room temperature.
3. Ensuring that sterile conditions are maintained, decant the supernatant and resuspend the pellet using a vortex mixer in 5 mL of BG-11. Next top-up the Falcon tube with 40 mL of BG-11 medium and spin-down the cells as in **step 2**.
4. Repeat the wash in **step 3** two additional times to remove any traces of glucose.
5. After the final wash resuspend the pellet in 5 mL of BG-11 using a vortex mixer and then, taking care to maintain sterile conditions, remove an aliquot of cells to determine the $OD_{730\ nm}$ (typically a 1 in 100 dilution is required to obtain an accurate reading).
6. Prepare culture flasks by pouring in 150 mL of BG-11 and adding the appropriate antibiotics. Next add the cells so that the starting $OD_{730\ nm}$ in the flask is approx 0.05. Measure the exact $OD_{730\ nm}$ and record this as the zero time point.
7. Attach the flasks to an aquarium pump and ensure that all cultures are bubbling evenly. Measure the $OD_{730\ nm}$ of the cultures every 12 or 24 h for 7 d (*see* **Note 21**).

3.6.2. Oxygen Evolution

The overall rate of photosynthesis, the rate of photosynthetic electron transport through the thylakoid membrane, as well as the activities of both photosystems can be assayed using a Clark-type oxygen electrode and an FLS1 light source supplied by Hansatech, UK. Saturating actinic light is required for these measurements and this can be experimentally determined by measuring activity as a function of light intensity with the aid of neutral density filters. It is recommended that the actinic light be passed through a Melles Griot OG 515 sharp cutoff yellow glass filter as this reduces photoinactivation of the samples. Activity measurements should be performed at 30°C in BG-11 containing 25 m*M*

HEPES-NaOH (pH 7.5). The chlorophyll *a* concentration should be between 10 and 20 µg/mL and the chlorophyll *a* should be extracted with methanol. The chlorophyll *a* determinations can be performed according to MacKinney ***(8)***.

The overall rate of photosynthesis can be measured by adding 10 m*M* $NaHCO_3^-$ from a fresh 1 *M* $NaHCO_3^-$ solution. Whole chain electron transport can be assayed by adding 2 m*M* methyl viologen from a 100 m*M* methyl viologen stock solution. This reaction will measure oxygen uptake and the reader is referred to ref. ***9*** for a complete discussion of the relationship between oxygen uptake and electron transport. A PSI partial reaction can be measured in the presence of 1 m*M* ascorbic acid and 1 m*M* 2,3,5,6-tetramethyl-*p*-phenylenediamine as electron donor (*see* **Note 22**). For this reaction 2 m*M* methyl viologen should be present as the electron acceptor and 20 µ*M* diuron should be present to block PSII activity. To measure PSII activity the electron acceptor should be 1.0 m*M* $K_3Fe(CN)_6$ with 0.2 m*M* 2,5-dimethyl-*p*-benzoquinone added to mediate between PSII and the non-penetrating $K_3Fe(CN)_6$. The recommended stock solutions are 250 m*M* for $K_3Fe(CN)_6$ and 50 m*M* for 2,5-dimethyl-*p*-benzoquinone (*see* **Note 22**).

3.7. Registering a Mutant With CyanoBase

To register a new mutant at CyanoBase go to http://www.kazusa.or.jp/cyano/ and select CyanoMutants. Select *Registration* and complete the requested details. To view other entries either select *Submission List* or use the keyword search.

3.8. Extending the Mutageneis System

The flexibility of *Synechocystis* PCC 6803 as a model organism for the study of gene knockouts is not restricted to the antibiotic-resistance markers identified in **Table 3**. By using combinations of the antibiotic-resistance cassettes, multiple genes can be inactivated in a single strain ***(10–12)***. Additionally, markerless deletions can be obtained in a two-step process using the *sacB* gene isolated from *Bacillus subtilis.* This gene encodes levansucrase which catalyzes the breakdown of sucrose to glucose and fructose derivatives ***(13).*** Expression of this gene has been shown to be lethal to *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 ***(14,15)***. In the first transformation *sacB*, in tandem with a selectable marker, is introduced into *Synechocystis* PCC 6803 with flanking sequences to replace the target gene. The resulting strain is both resistant to the antibiotic but sensitive to growth on 5% sucrose. In the second step, the strain is transformed with a construct that contains only the flanking sequences up-stream and down-stream of the inserted *sacB* and antibiotic-resistance cassette. The markerless deletion mutant obtained is able to grow on sucrose but is now sensitive to the antibiotic.

Synechocystis PCC 6803 has also been widely used for protein structure/function studies through the application of oligonucleotide-directed mutagenesis. For these experiments the target gene must first be replaced by an antibiotic-resistance cassette and full segregation demonstrated so as to ensure that all wt copies of the gene have been deleted. To introduce mutations a wt copy of the gene, together with an antibiotic-resistance cassette and flanking up-stream and down-stream sequences, are cloned into a vector, such as pGEM-T, that can be used for the generation of ssDNA. The resulting construct is transformed into *E. coli* strain CJ236. This strain is dUTPase and uracil-*N*-glycosylase deficient (dut^-ung^-) and therefore allows the stable incorporation of dU rather than dT into the single-stranded template. After the template has been used for in vitro mutagenesis reactions it is transformed into a dut^+ung^+ strain of *E. coli*, thus selecting against the parental strand. Details of this procedure can be found in refs. ***16–18*** and examples of their specific use with *Synechocystis* PCC 6803 may be found in refs. ***19*** and ***20*** (*see* **Note 23**). Further details of a range of mutagenesis-based strategies to study protein function in *Synechocystis* PCC 6803 have been described in Vermaas ***(5)***.

4. Notes

1. Strains can be requested by email at cyano@pasteur.fr and the website is http://www.pasteur.fr.
2. *Synechocystis* PCC 6803 is a mesophilic bacterium and is able to grow optimally between 30 and 34°C.
3. When cooled to room temperature a brown precipitate is formed and the bottle should be shaken before use. When 10 mL of 1 *M* TES-NaOH (pH 8.2) is added the amount of deionized water should be adjusted accordingly. The organism is basophilic and grows well between pH 8.0 and pH 10.0.
4. BG-11 plates can be stored at 4°C; however, when antibiotics are present storage periods should not exceed 4 wk.
5. Lighting can be provided by fluorescent tubes or metal halide bulbs.
6. In CyanoBase you can perform a protein or nucleotide BLAST search by pasting in your query sequence and this will identify the corresponding gene(s) in *Synechocystis* PCC 6803.
7. The nomenclature used to identify genes in CyanoBase begins with three letters followed by four numerals. The initial letter stands for the species, the second letter indicates gene length where "l" indicates longer than 100 codons and "s" less than 100 codons. The third letter indicates the direction of transcription on the circular genome map ("r" for right and "l" for left). The number corresponds to the sequential order of the putative open-reading frames.
8. When requesting nucleotide sequence be careful with respect to the direction of the gene given under the "Location" information. If the direction is complement then the nucleotide sequence will initiate with the larger number and terminate with a smaller number.

9. Ideally a single unique restriction site is selected for the introduction of an antibiotic-resistance cassette but if this is not possible a combination that results in an internal deletion of the gene may also be used providing that at least 0.4 kb is available for recombination at both ends.
10. Wash the laminar flow surfaces down with 70% ethanol, ethanol and flame your scissors and forceps, wear gloves when handling the filters (even rub ethanol over your gloves first). The Falcon tubes and test tubes used must also be sterile.
11. To accurately determine the $OD_{730\ nm}$ dilute an aliquot so the reading of the diluted sample is <0.4.
12. For high transformation efficiencies the $OD_{730\ nm}$ of the cells to be transformed should be between 2.0 and 3.0.
13. When using the glucose-tolerant strain to study mutations at the level of photosynthetic electron transport 5 m*M* glucose should be present in the BG-11 plates during the first 12 h. Subsequently, if specifically studying mutations in PSII proteins, 5 m*M* glucose and 20 μ*M* atrazine or diuron should be present in the plates to remove any selection pressure for the formation of PSII-specific revertants.
14. It is recommended that each BG-11 plate be divided into four quarters and four colonies picked with one streaked out in each quadrant. These can then undergo subsequent restreaks so that four replicates of each transformation are brought through the segregation steps.
15. The procedure in **Subheading 3.5.1.** for colony PCR may be enhanced by initially resuspending the colony in 200 μL TE containing 1% Triton X-100. The Triton X-100 can be subsequently removed by one or two chloroform extractions and a 5–10 μL aliquot of the upper aqueous phase added to the reaction. Heating the sample to 95°C for 2–4 min prior to extracting with chloroform has been carried out in some labs (e.g., http://microbiology.ucdavis.edu/meekslab/xpro6.htm).
16. In addition to the methods for genomic DNA isolation given in this section a rapid method for the isolation of DNA from cells on plates utilizing glass beads to break the cells is given in Vermaas *(5)*.
17. For a maximum yield of genomic DNA do not let the culture grow beyond an $OD_{730\ nm}$ of 3.0 before commencing this procedure. In this first step, centrifuge bottles compatible with either the Sorvall GSA or Beckman JA14 rotors are recommended.
18. Alternatively at **step 6** in **Subheading 3.5.3.** dry lysozyme can be added at 10 mg/mL followed by incubation at 37°C for 30 min. Following this incubation proteinase K and SDS are added to the suspension at a final concentration of 200 μg/mL and 0.5%, respectively. The sample is then incubated at 60°C for 30 min before proceeding to **step 8** in **Subheading 3.5.3.** This procedure can increase the yield of genomic DNA (S. Ermakova-Gerdes, personal communication).
19. Phenol-resistant polypropylene screw-capped tubes compatible with either the Sorval GSA or Beckman JA17 rotors are recommended for **steps 8–14** in **Subheading 3.5.3**.
20. In the case of the glucose tolerant strain of *Synechocystis* PCC 6803 the procedure outlined in **Subheading 3.6.1., steps 1–7** can be adapted for mixotrophic growth curves in the presence of 5 m*M* glucose or heterotrophic growth curves where, in addition to 5 m*M* glucose, 20 μ*M* atrazine or diuron are added.

21. Before taking a reading it is necessary to top-up the culture to 150 mL with sterile ddH_2O to correct for evaporation. In addition, when the cultures exceed an $OD_{730\ nm}$ of 0.4 it is necessary to dilute them to obtain an accurate reading.
22. The stock solutions for 2,3,5,6-tetramethyl-*p*-phenylenediamine and 2,5-dimethyl-*p*-benzoquinone are made up in ethanol.
23. Particularly in the case of membrane-spanning proteins DNA containing the promoter sequence should not be used for homologous recombination. *Synechocystis* PCC 6803 promoters may be recognized in *E. coli* and the expression of genes encoding hydrophobic foreign proteins may impose a selective pressure to inactivate expression. Thus upon reintroduction of the mutated gene into *Synechocystis* PCC 6803 the gene may be found to have been silenced. This problem can be circumvented by designing separate deletion-mutant strains for the 5' and 3' halves of the target gene.

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Gene Inactivation in the Cyanobacterium *Synechococcus* sp. PCC 7002 and the Green Sulfur Bacterium *Chlorobium tepidum* Using In Vitro-Made DNA Constructs and Natural Transformation

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Summary

Inactivation of a chromosomal gene is a useful approach to study the function of the gene in question and can be used to produce a desired phenotype in the organism. This chapter describes how to generate such mutants of the cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium tepidum* by natural transformation with synthetic DNA constructs. Two alternative methods to generate the DNA constructs, both performed entirely in vitro and based on the polymerase chain reaction (PCR), are also presented. These methods are ligation of DNA fragments with T4 DNA ligase, and megaprimer PCR.

Key Words: *Chlorobium*; cyanobacterium; gene inactivation; green sulfur bacterium; homologous recombination; megaprimer; natural transformation; polymerase chain reaction (PCR); *Synechococcus*.

1. Introduction

To inactivate a gene, typically a DNA construct is made in which the targeted gene is insertionally inactivated, or a portion of the gene is replaced, by a selectable marker conferring antibiotic resistance. The DNA construct must then be introduced into the cells of the organism in question (e.g., by transformation, conjugation, or electroporation), and those cells (i.e., transformants or transconjugates) in which the construct is incorporated into the genome by homologous recombination are selected by their antibiotic resistance. Some strains of bacteria are naturally transformable, which means that the cells can take up and incorporate exogenous DNA into their genomes by natural processes. Such bacteria can usually be readily transformed with DNA constructs

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made either in vivo (plasmids) or in vitro (polymerase chain reaction [PCR]-based) (*see* **Note 1**). In vitro-based methods overcome some of the problems associated with plasmid-based methods such as the cloning of toxic genes or the absence of suitable restriction sites for insertion of the selectable marker. In addition, any length of DNA can in principle be eliminated from the genome of the target organism with in vitro-based methods. For example, DNA constructs can be made that inactivate or delete several adjacent genes. Successful applications of in vitro techniques to generate gene inactivation constructs for transformation of cyanobacteria *(1,2)* and green sulfur bacteria *(3)* have been reported.

Two approaches to make DNA constructs for the insertional inactivation of genes by in vitro methods are described here: the ligation approach (**Subheading 3.1.**) and the megaprimer approach (**Subheading 3.2.**). The two methods are schematically depicted in **Fig. 1.** In both methods, the homologous DNA regions that flank the antibiotic resistance cartridge are made by PCR from genomic DNA (fragment **A** generated with primers **a1** and **a2** and fragment **B** generated with primers **b1** and **b2**; **Fig. 1**). However, the methods differ in how the two flanking DNA fragments are joined with the antibiotic resistance cartridge to obtain the final gene inactivation construct. For comments on the advantages and disadvantages of these methods, *see* **Note 2**. Several different antibiotic resistance markers are available for both *Synechococcus* sp. PCC 7002 and *C. tepidum* (*see* **Table 1**). The protocols presented under **Subheadings 3.1.** and **3.2.** use the *aadA* marker from plasmid pSRA81 as an example.

In the ligation approach (**Fig. 1A**), the **a2** and **b2** primers are designed so fragment **A** contains a *Pst*I restriction site and fragment **B** contains an *Eco*RI site. Plasmid pSRA81, which has an asymmetric polylinker, is then digested with *Pst*I and *Eco*RI to generate marker fragment **M** containing the resistance marker gene *aadA*, which confers streptomycin and spectinomycin resistance (*see* **Note 6**). The three fragments are mixed and ligated with T4 DNA ligase. The DNA mixture is electrophoretically separated on an agarose gel, and the desired gene inactivation construct (fragment **AMB**) is excised from the gel and purified. The **AMB** fragment is then either used directly, or amplified by PCR prior to its use, for transformation. (Enough AMB fragment may be generated by ligation alone such that subsequent amplification by PCR is unnecessary.)

In the megaprimer approach (**Fig. 1B**), the **a2** and **b2** primers are designed so approx 20 nucleotides at the 3' end are complementary to the genomic DNA containing the target gene and approx 20 nucleotides at the 5' end are complementary to a region on the pSRA81 plasmid containing the *aadA* marker. Megaprimer PCR can then be carried out using fragments **A** and **B** as megaprimers and pSRA81 as template to create the final gene inactivation construct. Several publications discuss the optimization of megaprimer PCR *(2,8,9)*.

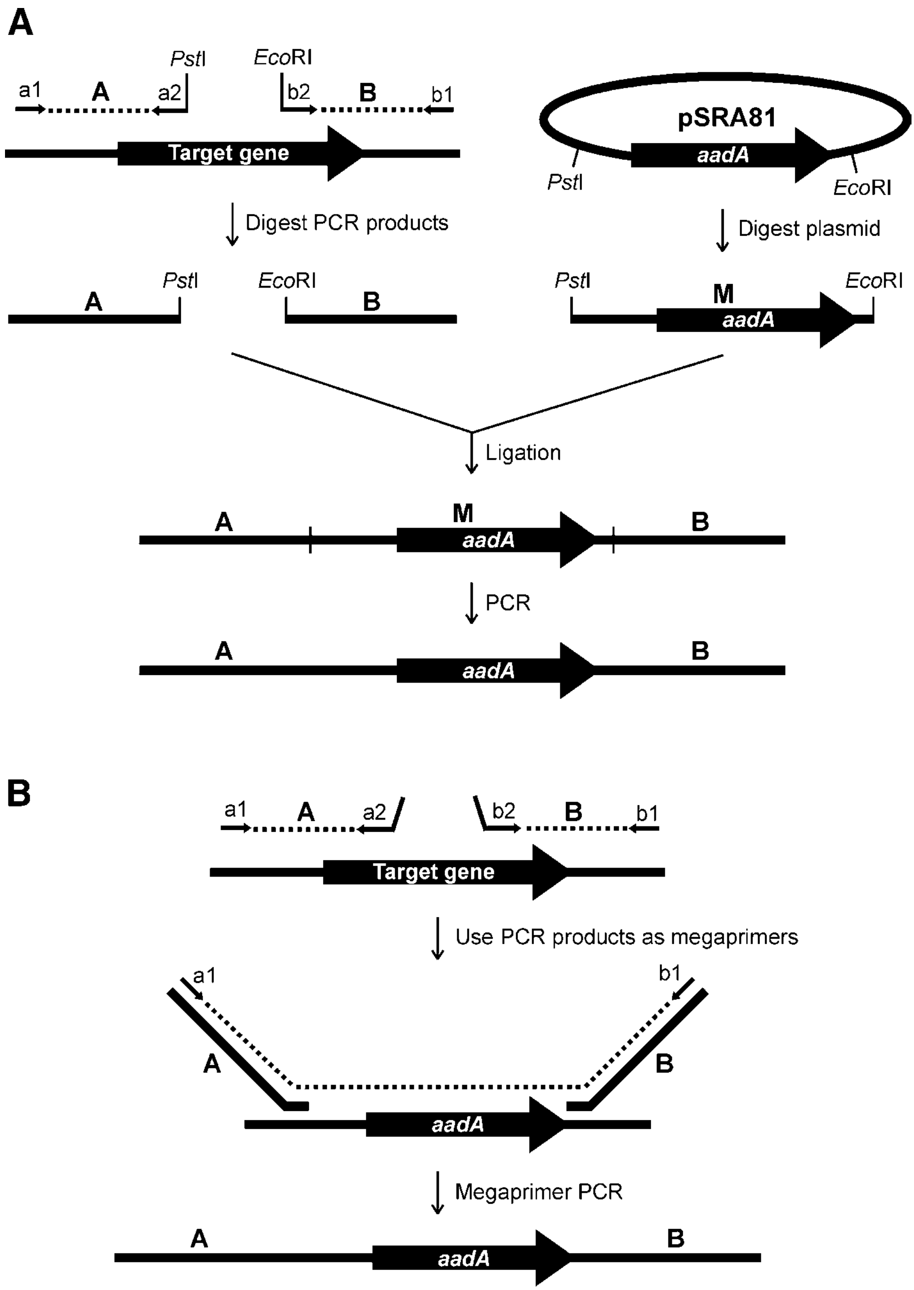

Fig. 1. Schematic depiction of two in vitro methods to generate DNA constructs for gene inactivation by homologous recombination: **(A)** the ligation method, and **(B)** the megaprimer PCR method. Arrows representing primers (**a1**, **a2**, **b1**, **b2**) point in the 5'→(3' direction and are shown parallel to homologous sequences. See text for details.

Table 1
Suitable Antibiotic Resistance Markers and Antibiotic Concentrations for Selection of *Synechococcus* sp. PCC 7002 and *C. tepidum* Transformants

Marker	*Synechococcus* sp. PCC 7002	*C. tepidum*	Plasmids
aacC1	Gm 50	Gm 100	pMS255, MS266 *(5)*
aadA	Sp 50	Sp 150 + Sm 300	pHP45Ω *(6)*, pSRA2, pSRA81 (*see* **Note 4**)
aphII	Km 200	Not suitable *(4)*	pRL161, pRL170 *(7)*
bla	Ap 5–50	Not suitable *(4)*	pBR322
cat	Cm 10	*See* **Note 3**	pRL409 *(7)*
ermC	Em 20	Em 2	pRL409 *(7)*

Numbers refer to the antibiotic concentration in μg/mL in liquid and solid media (*see* **Note 5**). Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin.

To transform the bacteria with the mutagenesis constructs, fresh cells are mixed with the DNA fragment. The cells are then incubated under nonselective conditions to permit DNA uptake, recombination of the inactivation construct into the genome, and expression of the antibiotic resistance marker gene. The cells are then transferred to selective conditions that only allow successful transformants to grow. The final step is to verify by PCR that the desired gene replacement has taken place and that the alleles have fully segregated in the isolated clone.

2. Materials

2.1. Preparation of Mutagenesis Constructs

1. Equipment and supplies for performing agarose gel electrophoresis of DNA (gel-casting system, power supply, agarose, ethidium bromide, TAE buffer, 5× loading buffer, etc.).
2. Equipment and supplies for performing PCR (PCR machine, aerosol-barrier tips, sterile water, etc.).
3. QIAquick® gel extraction kit (QIAquick® spin columns, QG buffer, PE buffer, EB buffer; QIAGEN, www.qiagen.com).
4. QIAquick® PCR purification kit (QIAquick® spin columns, PB buffer, PE buffer, EB buffer; QIAGEN, www.qiagen.com) (optional).
5. PLATINUM® *Pfx* DNA polymerase (polymerase, 10× amplification buffer, 50 m*M* $MgSO_4$ solution; Invitrogen, www.invitrogen.com) (*see* **Note 7**).
6. 2'-Deoxyribonucleoside 5'-triphosphate (dNTP) mixture for PCR.
7. Custom designed primers **a1**, **a2**, **b1**, and **b2** (*see* **Subheadings 3.1.1.** and **3.2.1.**).

8. Appropriate restriction enzymes and incubation buffers (*Pst*I and *Eco*RI are used as examples in **Subheading 3.1.**; only necessary for the ligation method).
9. T4 DNA ligase and incubation buffer (only necessary for the ligation method).
10. Plasmid containing an appropriate antibiotic resistance marker (plasmid pSRA81 is used as an example in **Subheadings 3.1.** and **3.2.**; *see* **Table 1**).
11. Supplies for ethanol precipitation of DNA (*see* **Note 8**) (optional).

2.2. Mutagenesis of Synechococcus *sp. PCC 7002*

1. Equipment for cultivation of *Synechococcus* sp. PCC 7002 in liquid and on solid medium (illuminated incubator or aquarium bath at 38°C, sterile gas delivery/bubbling device, etc.).
2. Liquid A^+ medium prepared with or without glycerol (*see* **Subheading 3.3.** and **Note 9**).
3. Plates with solid A^+ medium prepared with 1.5% (w/v) Bacto™ Agar (Becton Dickinson, Sparks, MD) and with or without glycerol (*see* **Subheading 3.3.** and **Note 9**).
4. Appropriate antibiotics (e.g., streptomycin and/or spectinomycin for the *aadA* marker).

2.3. Mutagenesis of C. tepidum

1. Equipment for cultivation of *C. tepidum* in liquid and on solid medium (anaerobic chamber, anaerobic jars [BBL GasPak™ 100 system; Becton Dickinson], CO_2-H_2 generator pack [BBL GasPak™; Becton Dickinson], illuminated incubator at 42°C, etc.).
2. Liquid CL medium (*see* **Subheading 2.4.2.**).
3. Plates with solid CP medium (*see* **Subheading 2.4.3.**).
4. Appropriate antibiotics (e.g., spectinomycin and streptomycin for the *aadA* marker).

2.4. Growth Media for C. tepidum

The liquid medium is stable for months. Plates with solid medium should be used within a few days after preparation. Cysteine is included in the solid medium to create reducing conditions, but may be excluded if it interferes with the desired growth conditions. The plates are most conveniently poured under aerobic conditions; however, the time that cysteine-containing medium is exposed to air should be minimized to prevent oxidation of the cysteine. Thioacetamide is used for generating H_2S gas inside the anaerobic jar. Both the liquid and the solid medium contain the redox-indicator resorufin (formed by degradation of resazurin) which is colorless when reduced and pink around neutral pH when oxidized. The described media are derived from **ref. *18***.

2.4.1. Stock Solutions

Store all stock solutions at 4°C.

1. CP/CL Salts A: 0.32 g of $Na_2{\cdot}EDTA{\cdot}2H_2O$, 5 g of $MgSO_4{\cdot}7H_2O$, 1.25 g of $CaCl_2{\cdot}2H_2O$, 10 g of NaCl, and distilled water up to 500 mL.

2. CP/CL Salts B: 12.5 g of NH_4CH_3COO, 10 g of NH_4Cl, 57.5 g of $Na_2S_2O_3{\cdot}5H_2O$, and distilled water up to 500 mL.
3. CP/CL Buffers: 12.5 g of KH_2PO_4, 52.5 g of MOPS (3-[*N*-morpholino]propane sulfonic acid), and distilled water up to 500 mL.
4. Vitamin B_{12} (1 mg/mL): 20 mg of vitamin B_{12}, and distilled water up to 20 mL. Filter-sterilize this solution.
5. Resazurin (10 mg/mL): 0.2 g of resazurin (sodium salt), and distilled water up to 20 mL.
6. *Chlorobium* trace elements: 5.2 g of EDTA, 190 mg of $CoCl_2{\cdot}6H_2O$, 100 mg of $MnCl_2{\cdot}4H_2O$, 1.5 g of $FeCl_24H_2O$, 6 mg of H_3BO_3, 17 mg of $CuCl_2{\cdot}2H_2O$, 188 mg of $Na_2MoO_4{\cdot}2H_2O$, 25 mg of $NiCl_2{\cdot}6H_2O$, 70 mg of $ZnCl_2$, 30 mg of $VoSO_4$, 2 mg of $Na_2WO_4{\cdot}2H_2O$, 2 mg of $NaHSeO_3$, and distilled water up to 1 L ***(10)***.

2.4.2. Liquid CL Medium

1. Prepare a solution by mixing in order: 800 mL of distilled water, 20 mL of CP/CL Salts A, 20 mL of CP/CL Salts B, 20 mL of CP/CL Buffers, 1 mL of *Chlorobium* trace elements, 50 µL of resazurin (10 mg/mL), and 20 µL of vitamin B_{12} (1 mg/mL). Adjust the volume to 1 L with distilled water. Add and keep a magnetic stir bar in the bottle. Autoclave the solution at 121°C for 20 min.
2. Prepare a sulfide-bicarbonate solution by dissolving 0.6 g of large, clear crystals of $Na_2S{\cdot}9H_2O$ and 2 g of $NaHCO_3$ in about 50 mL of distilled water in an almost completed-filled screw-capped tube. (Do not store this solution—use it immediately after preparation.) Add the solution through a 0.22-µm filter to the cooled medium from **step 1.** (This may be done under aerobic conditions but the bottle should be closed immediately after addition and transferred to an anaerobic chamber.)
3. The pH of the medium is usually within the desired range of 6.9–7.0 after addition of the sulfide-bicarbonate solution; if not, adjust pH accordingly with 1 *M* HCl or 1 *M* NaOH.

2.4.3. Solid CP Medium

1. Prepare a solution by mixing in order: 800 mL of distilled water, 20 mL of CP/CL Salts A, 20 mL of CP/CL Salts B, 20 mL of CP/CL Buffers, 1 mL of *Chlorobium* trace elements, 50 µL of resazurin (10 mg/mL), 20 µL of vitamin B_{12} (1 mg/mL), and 0.36 g of cysteine. Add and keep a magnetic stir bar in the bottle. Adjust pH to 7.6 by slow addition of 10 *M* NaOH.
2. Add 15 g of Bacto™ Agar (Becton Dickinson, Sparks, MD) or 10 g of Phytagel (Sigma, St. Louis, MO) and adjust the volume to 1 L with distilled water. (It is not necessary to prewash these solidifying agents. Phytagel must be added slowly to rapidly stirring medium to prevent clumping.)
3. Autoclave the solution at 121°C for 20 min. Close the lid tightly immediately after the autoclave is opened to prevent oxidation of the cysteine. Let the medium cool to roughly 50°C before proceeding.
4. Add antibiotics from a liquid stock solution if necessary.

5. Pour the plates and let them cool for no longer than 20 min. (One liter of CP medium makes about 20 to 30 plates. Do not stack the plates as this slows solidification. Pouring may be done in a cold room to speed up solidification. Move the plates to an anaerobic chamber immediately after solidification. The plates are pink when prepared and turn colorless when kept in the anaerobic chamber overnight. The pH should be about 6.9–7.0 in the solidified medium; if the pH is out of this range, adjust the pH accordingly in **step 1** above.)
6. Incubation of the plates: Place the inoculated plates in a jar together with about 0.1 g of thioacetamide in a small disposable glass tube and an opened CO_2-H_2 generator GasPak. (Tip: open the GasPak 10 min before usage to allow the air to diffuse out.) Activate the thioacetamide by adding 1 mL of 1 *M* HCl and activate the GasPak by adding 10 mL of water. Rapidly close the jar, keep it dark for 2 h, and then incubate it in the light. (Tip: If the jar is kept inside the anaerobic chamber it is not necessary to include the palladium catalyst.)

3. Methods

3.1. Mutagenesis Construct Made by Ligation

3.1.1. Construction of Primers

Figure 1A shows the principle of designing the primers (**a1** and **a2** for fragment **A** and **b1** and **b2** for fragment **B**; *see* **Notes 1** and **10**). Primers **a2** and **b2** should contain the indicated restriction site (*see* **Note 11**). The lengths of the **A** and **B** fragments should be chosen such the **AMB** fragment can easily be identified and isolated from the ligation mixture, which will contain primarily **AA**, **BB**, and **AMB** ligation products in addition to smaller amounts of other ligation products and **A** and **B** fragments that have not ligated. For example, with an **M** fragment of 1.0 kb, a suitable size of the **A** and **B** fragments is 0.6–0.8 kb each, which makes the final **AMB** fragment about 2.2–2.6 kb. One may design the PCR primers such that fragments **A** and **B** have different sizes. Restriction endonuclease digestion of product **AMB** followed by agarose gel electrophoresis can then be used to verify that the final **AMB** construct has the expected composition.

3.1.2. Preparation of Fragments A and B

1. Synthesize fragments **A** and **B** by performing 100-μL PCR reactions. (For optimization of the reaction conditions, try two concentrations of Mg^{2+}: 1.0 and 1.5 m*M*; *see* **Note 12**.)
2. Identify one successful PCR reaction for fragments **A** and **B** by analyzing 5 μL of the reaction mixtures on a 0.8% (w/v) agarose gel.
3. Concentrate and desalt the DNA from 100 μL of PCR reaction mixture by ethanol precipitation (*see* **Note 8**) or purify the DNA on a QIAquick column and resuspend it in 50 μL of water or QIAGEN's EB buffer. This should yield several μg of each fragment.

4. Digest fragments **A** and **B** with the appropriate restriction enzyme. (For example, mix 50 µL of DNA solution, 10 µL of 10× reaction buffer, 50 U of enzyme, and sterile water up to 100 µL, and incubate at the appropriate temperature for 4 to 5 h.)
5. Concentrate and desalt the DNA by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in a total of 50 µL of 1× gel loading buffer.
6. Electrophorese the samples on a 0.8% (w/v) agarose gel, excise the bands corresponding to fragments **A** and **B**, purify each DNA fragment using a QIAquick column, and elute each fragment in 30 µL of QIAGEN's EB buffer.

3.1.3. Preparation of Fragment M

1. Digest an amount of plasmid corresponding to about 2 µg of fragment **M** (equivalent to 7 µg of pSRA81) with the appropriate restriction enzymes.
2. Concentrate and desalt the DNA by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in a total of 50 µL of 1× gel loading buffer.
3. Electrophorese the sample on an agarose gel, excise the band corresponding to fragment **M**, purify the DNA on a QIAquick column, and elute it in 30 µL of QIAGEN's EB buffer.

3.1.4. Preparation of Mutagenesis Construct by Ligation and PCR

1. Set up a ligation reaction containing 30 µL of fragment **A**, 30 µL of fragment **B**, 30 µL of fragment **M**, 20 U of T4 DNA ligase, and 20 µL of 10× ligase buffer. Add sterile water up to a total volume of 200 µL. Incubate at room temperature for 3 to 4 h.
2. Concentrate and desalt the DNA by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in a total of 30 µL of 1× gel loading buffer.
3. Electrophorese the sample on a 0.8% (w/v) agarose gel, excise the band corresponding to the **AMB** ligation product, purify it using a QIAquick column, and elute the DNA in 50 µL of QIAGEN's EB buffer. This should yield at least 1 µg of the final **AMB** product (*see* **Note 13**).
4. Amplify the **AMB** product by PCR using primers **a1** and **b1**. Use the PCR conditions stated in **Note 12** except use 2 µL of **AMB** solution as template and 5 U of PLATINUM *Pfx* DNA polymerase per 100 µL of reaction mixture, and use an extension time of 3 min at 68°C.
5. Concentrate and desalt the DNA by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in 50 µL of water or QIAGEN's EB buffer. (Purify the PCR product on an agarose gel as in **step 3** if undesired DNA fragments are present. DNA solubilized in either water or QIAGEN's EB buffer is suitable for addition to the cells for transformation.)

3.2. Mutagenesis Construct Made by Megaprimer PCR

3.2.1. Construction of Primers

Figure 1B shows the principle of designing the primers (**a1** and **a2** for fragment **A** and **b1** and **b2** for fragment **B**; *see* **Notes 1** and **10**). A nucleotide sequence is

Table 2
Examples of Nucleotide Sequences for Designing a2 and b2 Primers to be Used in Megaprimer PCR (*see* Subheading 3.2.1.)

Marker	Template	Primer type	Sequence	Amplified sequence (bp)
aacC1	pMS266	**a2**	5'-GGTCTCCACGCATCGTCA-***X***-3'	
		b2	5'-GTTACCACCGCTGCGTTC-***X***-3'	884
aadA	pHP45Ω,	**a2**	5'-GTTACCACCGCTGCGTTC-***X***-3'	981
	pSRA2, pSRA81	**b2**	5'-CAAGGTAGTCGGCAAATAATGT-***Y***-3'	
aphII	pRL161,	**a2**	5'-CTTTAGCAGCCCTTGCG-***X***-3'	1125
	pRL170	**b2**	5'-TGACGAGTTCTTCTGAGCG-***Y***-3'	

contained in the 5' end of the **a2** and **b2** primers to allow fragments **A** and **B** to bind to the template containing the antibiotic resistance marker. Examples of such nucleotide sequences that bind to plasmids containing an antibiotic resistance marker are shown in **Table 2** (*see* **Note 14**). For example, to make a construct containing the *aadA* gene, first design four primers such that primers **a1** and ***X*** generate fragment **A** and primers **b1** and ***Y*** generate fragment **B** (where all primers are about 18 to 22 nucleotides long). Add the nucleotide sequences listed in **Table 2** to ***X*** and ***Y*** to obtain the final primers **a2** and **b2**, which will be about 40 nucleotides long and have the sequence 5'-GTTACCACCGCTGCG TTC-***X***-3' and 5'-CAAGGTAGTCGGCAAATAATGT-***Y***-3', respectively. Illustrative examples of this kind of megaprimer PCR are available in the literature ***(2,3,9)***. Also *see* **Note 7**.

3.2.2. Preparation of Plasmid Template

1. Digest about 10 µg of pSRA81 with *Ahd*I, which cuts the plasmid once. (A linear DNA template usually gives a higher product yield in megaprimer PCR than a circular DNA template.)
2. Concentrate and desalt the DNA by ethanol precipitation (*see* **Note 8**) or purify the DNA on a QIAquick column, and resuspend it in 30 µL of water or QIAGEN's EB buffer.
3. Electrophorese 0.2 µL of the DNA solution on a 0.8% (w/v) agarose gel to estimate the DNA concentration.

3.2.3. Preparation of Megaprimers by PCR

1. Synthesize fragments **A** and **B** by performing 100-µL PCR reactions. (For optimization of the reaction conditions, try two concentrations of Mg^{2+}: 1.0 and 1.5 m*M*; *see* **Note 12.**)

2. Identify one successful PCR reaction for each of fragments **A** and **B** by analyzing 5 µL of the reaction mixtures on a 0.8% (w/v) agarose gel.
3. Concentrate and desalt the DNA from 100 µL of PCR reaction mixture by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in 50 µL sterile water or QIAGEN's EB buffer (*see* **Note 13**). (Purify the megaprimers by electrophoresis on an agarose gel if undesired DNA products are present.)
4. Electrophorese 1 µL of the DNA solutions on a 0.8% (w/v) agarose gel to estimate the DNA concentration.

3.2.4. Preparation of Mutagenesis Construct by Double Megaprimer PCR

1. Perform a 100-µL PCR reaction containing: 10 µL of 10× buffer, 0.3 m*M* of each dNTP, 1.5 m*M* of Mg^{2+}, 0.5 µ*M* of each primer **a1** and **b1**, about 0.5 µg of each megaprimer **A** and **B** (typically about 10 µL), 0.3 µg of template (typically 1–2 µL), and 5 U of PLATINUM *Pfx* DNA polymerase; temperature program for PCR: 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 3 min, and a final elongation step at 68°C for 10 min. (For optimization of the reaction conditions *see* **Note 15**.)
2. Concentrate and desalt the DNA by ethanol precipitation or purify the DNA on a QIAquick column and resuspend it in a total of 30 µL of 1× gel loading buffer.
3. Electrophorese the sample on a 0.8% (w/v) agarose gel, excise the band corresponding in size to the expected DNA product, purify it using a QIAquick column, and elute the DNA in 50 µL of QIAGEN's EB buffer. (DNA solubilized in either water or QIAGEN's EB buffer is suitable for addition to the cells for transformation.)

3.3. Transformation of Synechococcus *sp. PCC 7002*

For preparation of A^+ medium (containing 1.0 g/L of $NaNO_3$), refer to Steven et al. ***(11)*** When attempting mutagenesis of genes encoding proteins involved in photosynthesis, glycerol is frequently included in the A^+ medium to provide an alternative carbon source to CO_2 (*see* **Note 9**). Currently, most mutagenesis on *Synechococcus* sp. PCC 7002 involves the photosynthetic apparatus, therefore the protocol given here includes 10 m*M* glycerol in the A^+ medium. *Synechococcus* sp. PCC 7002 grows optimally at 38°C; however, plates with solid medium are most conveniently incubated at, or slightly above, room temperature (typically around 30°C). It is recommended to use pipets and pipet tips with an aerosol barrier when handling liquid cultures, sterile media, and gas mixtures to prevent contamination. Strains may be frozen and stored as described in **Note 16**.

1. Prepare an exponentially growing culture of *Synechococcus* sp. PCC 7002 at 38°C in liquid A^+ medium containing 10 m*M* glycerol and bubbled with sterile air supplemented with 1% CO_2. Harvest the cells and resuspend them in fresh medium to an $OD_{730\ nm}$ of 2 to 3.

2. Mix 1 to 5 µg of DNA and 0.8 mL of culture and incubate under strong illumination (about 250 µE m^{-2} s^{-1}) at 38°C for 5 h while gently bubbling with sterile air supplemented with 1% CO_2. (Keep the volume of the added DNA solution less than one-tenth of the culture volume.)
3. Spread the cell suspension over a plate with solid A^+ medium containing 10 m*M* glycerol and allow the cells to grow under moderate illumination (about 150 µE m^{-2} s^{-1}) at 30°C for about 3 d until a thin lawn of growth is visible.
4. Overlay the plate with sterile, melted 0.8% (w/v) agar in water containing antibiotic (not warmer than about 50°C) over the cells. Use about 3 mL per plate (diameter 9 cm) containing 40 to 50 mL of solid medium. (Adjust the amount of antibiotic added such that the final concentration is calculated for the entire volume of solid medium in the plate.)
5. Incubate the plate under the same conditions as in **step 3** until single colonies appear (at least 2 to 3 wk).
6. Transfer several colonies on fresh solid, A^+ medium containing 10 m*M* glycerol and the appropriate antibiotic and incubate again. Restreaking should be repeated until a homozygous isolate is obtained (two to three restreakings from a single colony is usually sufficient unless the gene product is required for viability or strongly selected for under the growth conditions employed).
7. For each clonal isolate, streak one colony in a small area (about 1 cm^2) on a fresh plate and incubate until a thick patch of cells appear. Use this patch of cells for verification of allele segregation (**Subheading 3.5.**). *See* **Note 17**.

3.4. Transformation of **Chlorobium tepidum**

Except for the centrifugation in **Subheading 3.4.**, **step 2**, all other manipulations from **step 2** to **step 7** should take place in an anaerobic chamber. Plates may be incubated at 40 to 42°C at a distance of about 30 to 60 cm from a 100 W incandescent light bulb and liquid cultures may be incubated at 42 to 48°C. It is recommended to use pipets and pipet tips with an aerosol barrier when handling liquid cultures and sterile media to prevent contamination. Strains may be frozen and stored as described in **Note 18**.

1. Prepare a culture of *C. tepidum* in late exponential growth phase (*see* **Note 19**).
2. Aliquot 0.5 mL of culture into sterile screw-capped microcentrifuge tubes, cap the tubes, and harvest the cells by centrifugation for 20 s. Discard the supernatant.
3. Add growth medium and DNA solution to a final volume of about 100 µL (containing a total of 0.1 to 1 µg of DNA) to a cell pellet and mix the suspension thoroughly by vigorous mixing. (Keep the volume of the added DNA solution less than one-quarter of the medium volume.) Also prepare a negative control in which growth medium but no DNA is added to an aliquot of cells.
4. Spot each suspension in a small area on separate non-selective CP plates and incubate the plates overnight. (The cells should visibly have grown after this incubation.)

5. Scrape up as much of the cell material as possible and streak the cells on antibiotic-containing CP plates. Incubate the plates from 4 d to 3 wk depending upon the growth defect anticipated for the mutation attempted (*see* **Note 20**).
6. Transfer several colonies to fresh antibiotic-containing CP plates and incubate again. Restreaking should be repeated until a homozygous isolate is obtained (three restreakings from a single colony is usually sufficient).
7. For each clonal isolate, transfer one colony to a tube containing 1 mL of growth medium and incubate the culture until dense growth has occurred. Then add 10 to 20 mL of additional growth medium. Use this culture for verification of allele segregation (**Subheading 3.5.**) (*see* **Note 17**).

3.5. Confirmation of Segregation by PCR

The two outermost primers used for making the gene inactivation constructs (**a1** and **b1**; **Fig. 1**) can be used for checking segregation of the attempted mutation (*see* **Note 10**). This is conveniently done if the distance between the **a2** and **b2** primers is much smaller than the length of the inserted antibiotic resistance marker. In this way, the PCR product generated with the **a1** and **b1** primers will be longer in the segregated, homozygous mutant than in the wild-type and heterozygous mutants and thus confirm segregation. Genomic DNA from *C. tepidum* may be isolated as described in ref. ***12***. Genomic DNA from *Synechococcus* sp. PCC 7002 may be isolated as described in **ref. *13***. Alternatively, PCR may be performed by adding 1 μL of washed cells (same as a dense culture) directly to a 50-μL PCR reaction mixture.

4. Notes

1. For successful natural transformation of *Synechococcus* sp. PCC 7002 and *C. tepidum* with PCR-based DNA constructs, the two regions of homologous DNA flanking the antibiotic resistance marker should be at least 500 bp each; higher transformation frequencies are observed with increasing lengths of the flanking DNA ***(4)***. In contrast, if the gene inactivation construct is made as a plasmid, flanks of as little as 100 bp each are sufficient when the homologous DNA is flanked by at least several hundred base pairs of vector DNA. If a plasmid is used for transformation, a higher transformation efficiency can be obtained in both *Synechococcus* sp. PCC 7002 and *C. tepidum* if the plasmid is linearized by digestion with a restriction enzyme prior to transformation. Details of parameters affecting transformation efficiency can be found in the literature ***(14–16)*** for *Synechococcus* sp. PCC 7002 (formerly *Agmenellum quadruplicatum* strain PR-6) and Frigaard and Bryant ***(4)*** for *C. tepidum*.
2. All primers needed in the ligation approach may be of standard, desalted quality. However, the **a2** and **b2** primers needed in the megaprimer approach are relatively long and should be of higher purity (e.g., PAGE or HPLC purified), and thus are relatively expensive. The ligation approach requires the availability of an

antibiotic resistance marker gene cloned into a plasmid with an appropriate polylinker (as in pSRA81, *see* **Notes 4** and **6**).

3. The *cat* marker from pRL409 that confers resistance to chloramphenicol in cyanobacteria does not function in *C. tepidum* ***(4)***. However, the *cat* marker from pGSS33 (similar to the *cat* marker in pBR325) has been reported to function in *C. tepidum* ***(18)***. This is probably owing to a difference in the promoter for the *cat* gene in pRL409 and pGSS33 ***(7)***. Chloramphenicol prevents growth of *C. tepidum* at concentrations of 5 to 30 μg/mL ***(4,18)***.
4. Plasmids pSRA2 and pSRA81 contain the *aadA* marker from pHP45Ω cloned in high-copy-number vectors and flanked by suitable polylinkers (N.-U. Frigaard and D. A. Bryant, unpublished) and are available from the authors upon request.
5. The growth of *C. tepidum* mutants in liquid cultures has been observed to be somewhat inhibited by the recommended concentrations of spectinomycin and streptomycin even though the cells contain the *aadA* marker. Therefore, it is recommended to measure growth rates of *C. tepidum* mutants in the absence of antibiotics.
6. The polylinker of pSRA81 containing the *aadA* gene is: *Hin*dIII-*Sph*I-*Pst*I-*Sal*I-***aadA***-*Xba*I-*Bam*HI-*Kpn*I-*Eco*RI (*see* **Note 4**). This polylinker allows other combinations of enzymes than *Pst*I and *Eco*RI to be used if desired or necessary.
7. The DNA polymerase used for PCR in **Subheadings 3.1.** and **3.2.** should be a proofreading polymerase with 3'→5' exonuclease activity such as the PLATINUM *Pfx* DNA polymerase (Invitrogen) to minimize introduced mutations. In addition, nonproofreading polymerases like *Taq* DNA polymerase nonspecifically add a nucleotide (mostly an adenosine residue) to the 3' end of PCR products, which may interfere with the priming ability of the megaprimers (fragments **A** and **B**) in the PCR in **Subheading 3.2.4.**, **step 1**.
8. Protocol for ethanol precipitation of DNA: Add 0.1 vol of 3 *M* sodium acetate, pH 5.0 and mix. Add 2.5 vol of cold ethanol (or 1 vol of cold isopropanol) and mix. Incubate at –20°C for 15 min. Pellet DNA by centrifugation for 10 min at 4°C. Wash the pellet with 700 μL room-temperature 70% (v/v) ethanol and centrifuge again for 5 min. Let the pellet dry and redissolve it in an appropriate volume of buffer. (For extraction of proteins and lipids prior to ethanol precipitation: add an equal volume of phenol:chloroform:isoamyl alcohol [25/24/1 by vol] and mix vigorously for 10 to 30 s. Centrifuge for 1 min at room temperature, and transfer the top [aqueous] phase to a fresh tube.)
9. Because A^+ medium contains no organic carbon source, *Synechococcus* may exhibit growth difficulties when mutated in the photosynthetic apparatus. Providing glycerol as alternative carbon source to CO_2 allows heterotrophic growth under such conditions. However, because the wild-type strain of *Synechococcus* sp. PCC 7002 is somewhat glycerol sensitive, a glycerol-tolerant strain must first be isolated by numerous transfers of large, single colonies on solid A^+ medium containing 10 m*M* glycerol. Keep in mind it may be worthwhile to attempt the mutagenesis of *Synechococcus* sp. PCC 7002 both in the absence and presence of glycerol to determine whether your attempted mutation affects photosynthetic carbon metabolism. (Tip: Serially subculturing the wild type in the presence of increasing glycerol

concentrations in liquid medium can speed up the isolation of a glycerol-tolerant strain.)

10. Primers are most conveniently designed using dedicated software. First design four primers **a1**, **b1**, ***X***, and ***Y***, each about 18 to 22 nucleotides long, such that **a1** and ***X*** generates fragment **A**, and **b1** and ***Y*** generates fragment **B** (a suitable size of fragment **A** and **B** is between 600 and 800 bp). Primers **a2** and **b2** are then obtained by modification of ***X*** and ***Y***, respectively, according to the method to be used (for ligation approach, *see* **Subheading 3.1.1.**, for megaprimer approach, *see* **Subheading 3.2.1.**). A suitable position of the **a2** primer is between the start codon and about the midpoint of the target gene. The distance between the **a2** and **b2** primers should be significantly different from the length of the inserted antibiotic resistance marker, since this will facilitate confirmation of segregation of the obtained mutants by PCR using the **a1** and **b1** primers (*see* **Subheading 3.5.**). It is therefore also advisable to check with dedicated software that the **a1** and **b1** primers are compatible.
11. The restriction site can conveniently be introduced anywhere between the middle of the primer and the 3' end. However, at least the first four bases from the 3' end should be homologous to the template DNA. Primers designed this way and for the purpose of ligation of PCR products need not be of highly purified quality; primers of standard, desalted quality are much cheaper and will suffice. A general tip about modifying the sequence of a primer: For every base that is changed from a homologous base to a heterologous base, add one or two homologous bases to either end of the primer.
12. Composition of a typical PCR reaction using genomic DNA for amplification of fragments **A** and **B**: 10 µL of 10× buffer, 0.3 m*M* of each dNTP, 1 m*M* $MgSO_4$ 0.5 µ*M* of each primer, 100 ng of genomic DNA, 2.5 U of PLATINUM *Pfx* DNA polymerase, sterile water to 100 µL. Typical temperature program: 94°C for 4 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 1 min, and a final elongation step at 68°C for 10 min.
13. Care must be taken in purifying DNA to be used in PCR. Many contaminants (including ethanol) from gel extraction kits inhibit PCR. It is recommended to let QIAquick columns dry 5 min after washing with QIAGEN's PE buffer to allow residual ethanol to evaporate before adding QIAGEN's EB buffer.
14. Alternative 5'-end sequences for designing **a2** and **b2** primers that will bind to other plasmids can be obtained as follows: Design a pair of primers (each 16 to 22 nucleotides long) which will amplify the desired antibiotic resistance marker (including promoter if desired). The 5'-end sequences needed for **a2** and **b2** then corresponds to the complement sequences of this primer pair.
15. The concentration of megaprimers (fragments **A** and **B**) is not critical for successful megaprimer PCR but should be similar to each other and roughly about 250 to 1500 ng each per 100 µL reaction mixture. The template concentration is more critical and can be varied between 200 and 500 ng per 100 µL reaction mixture. For optimization of the Mg^{2+} concentration try 1.5 and 2.0 m*M*. (In some instances, it is possible to avoid **Subheading 3.2.3.**, **step 3**, by using only 5 ng of genomic

DNA in **Subheading 3.2.3.**, **step 1** and adding 5 µL of the crude PCR reaction mixture from **Subheading 3.2.3.**, **step 1** directly to the megaprimer PCR reaction in **Subheading 3.2.4.**, **step 1.**)

16. Freezing and storage of wild type and mutant strains of *Synechococcus* sp. PCC 7002 can be performed as previously described ***(17)***: Harvest a late-exponential culture and resuspend the cells in fresh A^+ medium containing 10 m*M* glycerol and 5% (v/v) methanol to one-tenth of the original culture volume. Aliquot into 1-mL samples in cryovials, place the cryovials in a refrigerated "Mr. Frosty" freezing container (Nalgene, Rochester, NY) and transfer the container to –80°C, (this device allows slow freezing). To revive, rapidly melt a culture by placing the cryovial in water at 35°C, harvest the cells by centrifugation, and resuspend in fresh A^+ medium containing 10 m*M* glycerol. Keep the culture in the dark at 38°C for a day before incubating under dim light.
17. Occasional reverification of the genotype of mutant strains is advisable, especially when the mutant does not have an easily discernible phenotype and since contamination may thus be difficult to discern by the visual appearance of the culture in liquid or on plates.
18. Freezing and storage of wild-type and mutant strains of *C. tepidum* can be performed as follows: Add 0.25 vol of 50% (v/v) glycerol to a late-exponential culture, aliquot into 1-mL samples, and immediately transfer the tubes to –80°C. To revive, melt a portion of the culture with finger heat, then either streak approx 10 µL of culture on solid CP medium to obtain single colonies, or transfer 100 µL of culture to 1 mL of CL medium. The remaining glycerol stock may be transferred back to –80°C and revived several times.
19. Continuous transfers of *C. tepidum* in liquid cultures tend to cause a loss of high plating efficiency as well as accumulation of other spontaneous mutations. It is therefore recommended to keep an axenic, antibiotic-sensitive culture of *C. tepidum* with a high plating efficiency stored at –20°C and revive this culture whenever a transformation is to be done (*see* **Note 18**).
20. Colonies of nonsegregated and spontaneously resistant mutants may show up on selective plates after 3 to 4 d. Colonies of segregated mutants may take longer to appear and may be different in size and coloration.

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