

Reconstitution of the Light Harvesting Chlorophyll *a/b* Pigment-Protein Complex into Developing Chloroplast Membranes Using a Dialyzable Detergent¹

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ABSTRACT

Conditions were developed to isolate the light-harvesting chlorophyll-protein complex serving photosystem II (LHC-II) using a dialyzable detergent, octylpolyoxyethylene. This LHC-II was successfully reconstituted into partially developed chloroplast thylakoids of *Hordeum vulgare* var Morex (barley) seedlings which were deficient in LHC-II. Functional association of LHC-II with the photosystem II (PSII) core complex was measured by two independent functional assays of PSII sensitization by LHC-II. A 3-fold excess of reconstituted LHC-II was required to equal the activity of LHC developing *in vivo*. We suggest that a linker component may be absent in the partially developed membranes which is required for specific association of the PSII core complex and LHC-II.

In the chloroplasts of higher plants, most of the antenna pigments which sensitize PSII are bound to a complex of intrinsic membrane polypeptides called the LHC-II.² LHC-II contains Chl *a* and *b* and carotenoids which are capable of transferring exciton energy to PSII. It accounts for approximately 50% of the total Chl in fully developed thylakoid membranes of higher plants (33).

The genes encoding the apoproteins of LHC-II are located in the nucleus (9). LHC-II polypeptides are synthesized in the cytoplasm and transported into the chloroplast where a 4 kD transit sequence at the N-terminus is cleaved (30). Integration of LHC-II into the thylakoid membrane can be independent of the synthesis and functional assembly of the PSII core complex (13, 34). However, LHC-II must interact with PSII in order to function in transferring absorbed excitons to the reaction center. This interaction is structural as demonstrated by studies employing freeze-fracture electron microscopy of developing thylakoids. The appearance of LHC-II in those membranes was correlated with an increase in the diameter of PSII particles from 8 to 16 nm (2); this was interpreted to be the result of a structural and

functional association of the LHC-II with the PSII core complex.

Recently, LHC-II has been hypothesized to carry out a dynamic role in the distribution of absorbed energy between PSII and PSI (3). Under conditions which favor the excitation of PSII relative to PSI, a surface-exposed segment of the LHC-II protein is phosphorylated. Upon phosphorylation, a subpopulation of the LHC-II functionally detaches from the PSII core complex and diffuses laterally to the stroma lamellae (14). This mechanism allows the plant to adapt to changing light quality in full sun versus shade. The process requires that a pool of LHC-II exist which can detach from the PSII reaction center. It has been hypothesized that two populations of LHC-II are present in the thylakoid membrane, one static or permanently bound to PSII, and the other dynamic and able to alter its functional association (14).

The structural and functional interaction of LHC-II and PSII core complexes thus appears to be an intricate process with regulatory mechanisms controlling their association other than simple collision. To study this association *in vitro* we have characterized a reconstitution system which allows us to manipulate the ratio of LHC-II:PSII in the membrane. The reconstitution was initially limited by the use of Triton X-100 to isolate LHC-II. Triton-solubilized preparations are inappropriate for reconstitution because the detergent is difficult to remove from solution during reconstitution. This paper describes the isolation and reconstitution of LHC-II in a dialyzable detergent, OPOE, which overcomes these problems.

MATERIALS AND METHODS

Octylpolyoxyethylene. OPOE was received as a gift from Dr. M. Schindler of Michigan State University. The synthesis of this detergent was previously described (28). The final product is a mixture of 3 to 12 oxyethylene units per octane. The critical micelle concentration of the components of the mixture ranges from 6.6 to 17.4 mM.

Preparation of LHC-II. *Hordeum vulgare* var Morex (Michigan Seed Foundation, East Lansing, MI 48823) was grown in growth chambers at 21°C under 16 h photoperiod. The LHC-II isolation is based on that of Burke *et al.* (5). Chloroplasts were isolated by homogenizing about 50 g of leaf tissue in 300 ml of a solution of 0.1 M Tricine-NaOH (pH 7.8) and 0.4 M sorbitol at 4°C. The homogenate was filtered through eight layers of cheese-cloth and centrifuged at 3,000g for 10 min. The pellet was resuspended in 200 ml of a solution of 0.1 M sorbitol and 0.75 mM EDTA, and centrifuged at 20,000g for 5 min. The pellet was washed twice more by resuspending in 0.1 M sorbitol and centrifuging at 20,000g. The final pellet was resuspended in distilled

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²Abbreviations: LHC-II, light harvesting chlorophyll *a/b* pigment-protein complex serving Photosystem II; OPOE, octylpolyoxyethylene; IML, intermittent light; DCPIP, 2,6-dichlorophenol-indophenol; DPC, diphenyl carbazide.

H₂O containing 0.1 mM DTT. Chl concentration was determined by the method of MacKinney (19). The thylakoids were diluted to a final concentration of 0.8 mg Chl/ml and OPOE was added to a final concentration of 1.25% (w/v). This mixture was stirred at 21°C for 30 min in the dark. Unsolubilized membranes were removed by centrifuging the mixture at 41,000g for 30 min. Eight ml of the supernatant was loaded onto each of six 3.4 to 34% (w/v) linear sucrose density gradients (38 ml total gradient volume) containing 0.25% OPOE (w/v) and centrifuged at 100,000g in a swinging bucket rotor for 16 h. After centrifugation, the tubes were exposed to high intensity side illumination from a microscope lamp. A fraction of approximately 5 ml in the top half of the gradient which showed high Chl fluorescence was collected. Purified LHC-II was aggregated from this solution by adding MgCl₂ to a final concentration of 5 mM, and stirring the solution for 10 min at 21°C in the dark. Quenching of the Chl fluorescence in the solution during the stirring provided visible evidence of aggregation. The aggregated LHC-II was then removed by centrifugation at 18,000g for 10 min. The pellet was resuspended in about 20 ml of a solution containing 10 mM Tricine-NaOH (pH 7.8) and 10 mM NaCl. Magnesium ions were removed by adding 10 mM EDTA (pH 7.8) and stirring the solution for 10 min at 4°C in the dark. LHC-II was recovered by centrifugation at 18,000g for 10 min. The EDTA wash was repeated, and the final pellet was resuspended in a solution containing 10 mM Tricine-NaOH (pH 7.8) and 10 mM NaCl. The aggregated LHC-II was dispersed by diluting it to 0.5 mg Chl/ml, adding OPOE to a final concentration of 0.6% (w/v), and stirring for 10 min at 21°C in the dark. Dispersion of the aggregated LHC-II was accompanied by a visually detectable increase in Chl fluorescence. This LHC-II/OPOE solution was used in all reconstitution experiments discussed in this paper, and is simply referred to as the LHC-II solution.

Intermittent Light Treatment and Isolation of Thylakoid Membranes. Barley seedlings which were grown for 7 d in complete darkness were exposed to IML cycles of 2 min light, 118 min dark for 48 h. Thylakoid membranes were isolated from these seedlings using a modification of the method of Mills and Joy (22). Fifty g of leaf tissue was homogenized in 230 ml of a solution containing 50 mM Tricine-NaOH (pH 7.8), 1 mM MgCl₂, 2 mM EDTA (pH 7.8), 330 mM sorbitol, and 0.1% (w/v) BSA at 4°C. The homogenate was filtered through 12 layers of cheese-cloth to remove cell fragments. Plastids were pelleted through 40% Percoll (v/v) in the homogenization buffer by centrifugation at 4,000g for 3 min. The pellet was then resuspended in a low osmotic solution (10 mM Tricine-NaOH [pH 7.8], 10 mM NaCl, 0.1% BSA) to lyse intact chloroplasts, and centrifuged at 12,000g for 10 min. The pellet was resuspended in 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, and 100 mM sorbitol. Chl concentration was estimated using the method of MacKinney (19) and the Chl *a*:Chl *b* ratio was measured by the low temperature method of Boardman and Thorn (4). An extra extraction of the Chl with anhydrous ether was included in that procedure to ensure that the sample was anhydrous before the addition of ethanol (see discussion in Haworth *et al.* [11]).

Reconstitution of Thylakoids with LHC-II. Two methods were used to induce the reconstitution of isolated LHC-II into the thylakoid membranes: dialysis and freeze-thaw. For dialysis-induced reconstitution, equal volumes of solubilized LHC-II and thylakoid membranes were mixed so that the ratio of LHC-II Chl:thylakoid Chl was 2:1 (w/w). Total Chl concentrations were 300 µg/ml in the 2:1 reconstituted samples and 100 µg/ml in the thylakoid membrane control samples. The mixture was dialyzed in a solution of 10 mM Tricine-NaOH (pH 7.8) and 10 mM NaCl for 12 h.

The freeze-thaw induced reconstitution was carried out using the method of Ryrie *et al.* (29). Immediately before reconstitu-

tion, the LHC-II solution was sonicated for 30 s in a bath sonicator. Equal volumes of LHC-II and thylakoid membranes were mixed so that the ratio of LHC-II Chl:thylakoid Chl was 2:1 or 1:1 (w/w). Total Chl concentrations were 125 µg/ml in the thylakoid and LHC-II control samples, 250 µg/ml in the 1:1 reconstituted, and 375 µg/ml in the 2:1 reconstituted samples. The solution was mixed thoroughly and rapidly frozen by immersing the tube containing the solution in liquid N₂. The mixture was allowed to thaw slowly in a 21°C water bath and was further sonicated for 30 s. Control samples were prepared for all measurements by diluting LHC-II or thylakoid membranes to the same final volume as the reconstitution mixture and treating the sample to the reconstitution treatment.

Density Gradient Centrifugation. Reconstituted membranes and control samples were loaded on 34 to 68% (w/v) sucrose density gradients and centrifuged at 250,000g for 20 h in a swinging bucket rotor. The gradients were fractionated from the top into 20 drop samples. The Chl concentration of each fraction was estimated by measuring *A* at 675 nm. Absorption spectra from 620 to 720 nm were recorded for the fractions of interest.

Polyacrylamide Gel Electrophoresis. PAGE was performed using the method of Laemmli (15). Gradient gels of 11 to 17% acrylamide were run at 4°C as described previously (32).

Measurement of Chlorophyll Fluorescence Transients. Chl fluorescence was measured with a photodiode positioned at 90° relative to an intense blue actinic beam (Corning Filter no. 4-96), as described previously (27). Excitation light intensity was 200 µE and constant for all experiments. The photodiode was protected from scattered blue light by a red cutoff filter (Corning Filter No. 2-64). Total Chl in the 2 ml reaction volume was always less than 10 µg, with thylakoid Chl held at 2.5 µg. The reaction mixture also contained 10 µM DCMU or Diuron and 10 mM NH₄OH. The voltage output of the photodiode was monitored on a Nicollet model 206 digital oscilloscope. The half-time of the variable fluorescence induction transient was determined from the recorded traces.

Measurement of Photosynthetic Electron Transport. PS II-dependent electron transport activity was measured using 0.03 mM DCPIP as electron acceptor and 1.0 mM DPC as electron donor. The reaction mixture contained the uncouplers NH₄Cl (1 mM) and gramicidin (10⁻⁷ M), and the reaction was monitored spectrophotometrically at 580 nm as previously described (6). The actinic light was filtered through narrow band-pass filters at 650 ± 6 nm and 684 ± 5 nm, preferentially exciting Chl *b* and Chl *a*, respectively. The exciting light intensity was 12 µE at 650 nm and 4 µE at 684 nm which was well below saturating intensities. In some samples, 10 µM DCMU was added to block PSII-dependent electron transport. In all samples thylakoid Chl was held at 1.25 µg/ml. Final Chl concentrations were: control thylakoids, 1.25 µg/ml; 1:1 reconstituted, 2.5 µg/ml; 2:1 reconstituted, 3.75 µg/ml.

Measurement of Chl Fluorescence and Absorption Spectra. Chl fluorescence spectra were measured on a SLM model 4800 spectrofluorometer linked to a HP 85 desktop computer. Samples containing 10 µg Chl/ml were prepared in 66% glycerol and frozen in liquid N₂ immediately prior to analysis. Excitation was at 440 nm. Chl fluorescence emission was recorded between 650 and 800 nm. Spectra were not corrected for instrument response.

Absorption spectra were measured on an SLM-Aminco DW-2c spectrophotometer. Chl concentration of the samples was 10 or 50 µg/ml.

RESULTS

Isolation of LHC-II. LHC-II was isolated from barley thylakoids using OPOE to solubilize the membranes. The preparation contained three polypeptides of 27, 25, and 24 kD (Fig. 1, lane B). A similar polypeptide pattern is found for LHC-II isolated

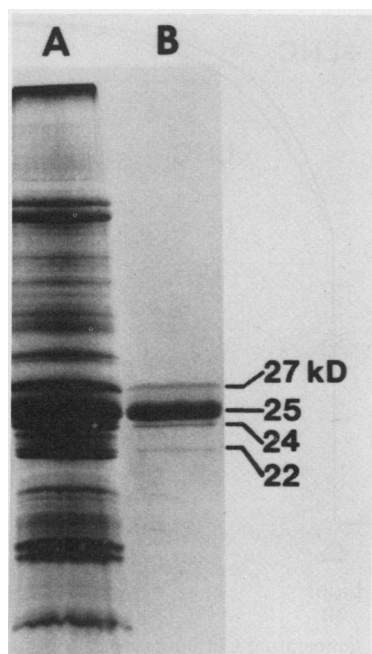


FIG. 1. SDS-PAGE of LHC-II purified from barley thylakoid membranes with OPOE. Lane A, barley thylakoids; lane B, LHC-II (1.5 μg Chl).

from barley using Triton X-100. In contrast, LHC-II isolated from pea thylakoids with either OPOE or Triton X-100 contains four polypeptides of 28, 26, 25.5, and 24.5 kD (data not shown). A 22 kD polypeptide was also present as a minor constituent in LHC-II prepared using OPOE. The relative amount of this protein varied between preparations. It has not been reported in previous studies of LHC-II. We believe it does not represent a degradation product since the protein does not appear in Triton X-100 derived LHC-II prepared under similar conditions, nor does it change in amount during sample aging.

Absorption spectra of barley thylakoids and OPOE-derived LHC-II are shown in Figure 2. The Chl *a*:Chl *b* ratio in isolated LHC-II is 1.15. The large amount of Chl *b* in the preparation is reflected by the absorption at 655 and 475 nm (Fig. 2B). The red absorption maximum shifts slightly when LHC-II is dispersed by treatment with EDTA and 0.6% OPOE (Fig. 2C). Low temperature Chl fluorescence spectra of similar samples are shown in Figure 3. The lack of a fluorescence peak at 735 nm in the isolated, aggregated LHC-II indicates that the sample was free of contaminating PSI (Fig. 3B). This LHC-II from barley had two strong emission maxima, at 681 and 700 nm. The strong 700 nm emission was not observed in LHC-II isolated from pea thylakoids using Triton X-100 (23) or OPOE (data not shown). However, this emission has been previously reported in LHC-II isolated from barley thylakoids using Triton X-100 (7), and is, therefore a characteristic of barley LHC-II rather than a side effect of solubilization in OPOE.

The 700 nm fluorescence emission component in aggregated LHC-II is decreased by the addition of detergent to the preparation (Fig. 3C). This type of change (the loss of the 695–700 nm emission component upon addition of detergent) has previously been attributed to disassociation of the LHC-II (23, 29). Further treatment of the sample in Figure 3C with Triton X-100 (up to 1.5%, w/v) eliminated the 700 nm shoulder completely (data not shown). The 700 nm emission of the aggregated LHC-II is much more pronounced with respect to the 681 emission in OPOE preparations than in any Triton X-100 derived samples we have prepared. We conclude that this is due to the fact that OPOE has a lower critical micelle concentration than Triton X-100 and is,

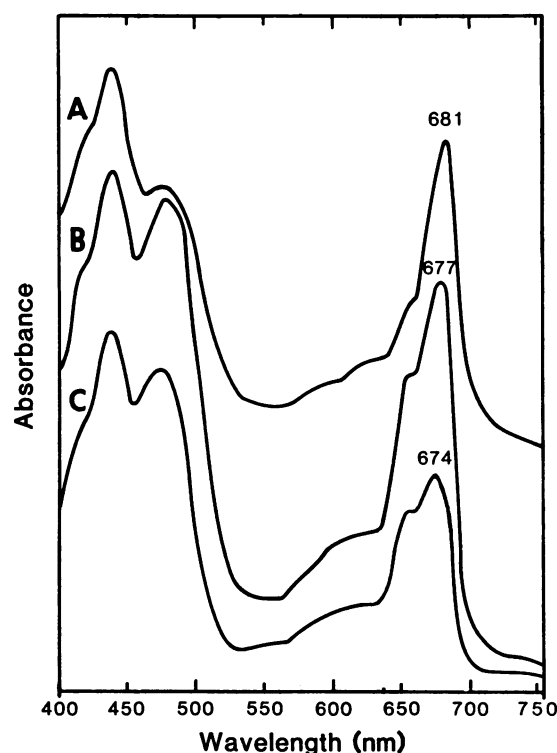


FIG. 2. Absorption spectra of LHC-II preparations. A, Barley thylakoid membranes (10 μg Chl/ml); B, isolated LHC-II aggregated by Mg^{2+} treatment (50 μg Chl/ml); C, isolated LHC-II dispersed by EDTA washes and addition of 0.6% OPOE (10 μg Chl/ml).

therefore, more readily removed from the isolated LHC-II. More extensive removal of the detergent would allow for greater aggregation of the protein with the resulting increase in 700 nm emission.

Dialysis Induced Reconstitution. Reconstitution of LHC-II into thylakoids isolated from partially developed barley seedlings (etiolated, then exposed to IML) was initially attempted using dialysis to remove the detergent and induce reconstitution. LHC-II in 0.6% OPOE was mixed with an equal volume of thylakoid membranes and dialyzed for 12 h. This time period was sufficient to aggregate a control sample of isolated LHC-II, as judged by Chl fluorescence quenching, to the same level as that induced by Mg. Dialysis treatment resulted in a loss of approximately 15% of the Chl in the sample subjected to dialysis. This loss was probably due to further solubilization and denaturation of some Chl complexes in the thylakoid during the exposure to OPOE in the 12 h of dialysis.

Functional interaction of the reconstituted antenna with the PSII core complexes was estimated by measuring the induction kinetics of room temperature Chl fluorescence transients. In the presence of DCMU, which blocks PSII electron transport from the primary to the secondary stable electron acceptors, the rate of fluorescence increase to its maximum level is proportional to the intensity of the exciting light and to the size of the antenna associated with the reaction center. As more antenna Chl is connected to each reaction center, the rate of exciton migration into that center is increased, Q_A is more rapidly reduced, and fluorescence reaches the maximum level more rapidly than it does in reaction centers with less functional antenna. Figure 4 shows a reproduction of fluorescence transients (in the presence of DCMU) in control (-LHC) and reconstituted (+LHC) thylakoid membranes. These fluorescence transients are compared by measuring the time required to reach half of the maximal level.

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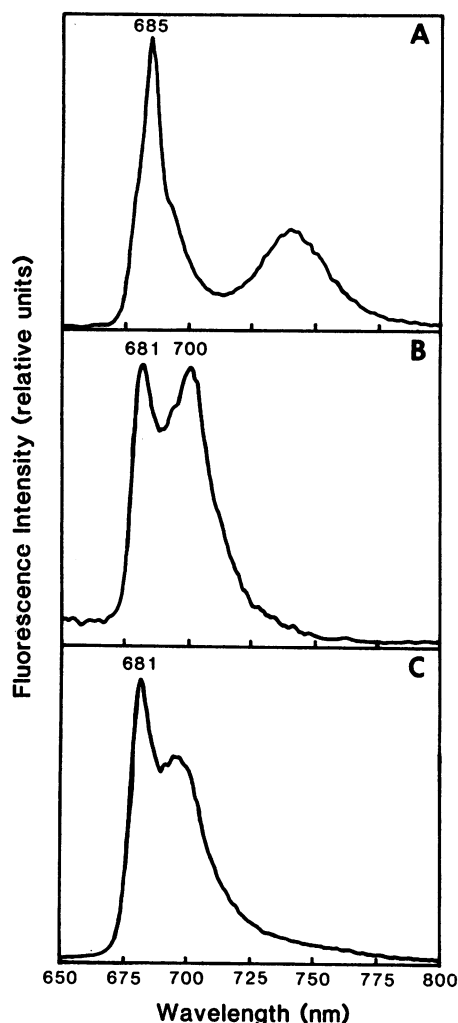


FIG. 3. Chl fluorescence emission spectra at 77°K of LHC-II preparations. A, Barley thylakoid membranes; B, isolated, aggregated LHC-II; C, isolated, dispersed LHC-II.

fluorescence induction in the dialysis-reconstituted membranes are presented in Table I. The $t_{1/2}$ of the control thylakoids (dialyzed in the absence of LHC-II) was 48.2 ± 1.5 ms. The unreconstituted mixture of LHC-II and thylakoids (mixed immediately before measurement) exhibited a $t_{1/2}$ equal to the control thylakoids. The dialysis-reconstituted mixture had a $t_{1/2}$ of 37.8 ± 1.9 ms, a decrease of 22% from the control. Thus, LHC-II was functionally interacting with the PSII in the reconstituted membranes. The amplitude of the variable fluorescence in the control, dialyzed thylakoids and the reconstituted membranes was equivalent indicating that a similar amount of active PSII was present in both samples.

Freeze-Thaw Reconstitution. LHC-II and thylakoid membranes were also reconstituted using a single freeze-thaw and brief sonication. The Chl fluorescence transient analysis is shown in Table I. A 2:1 reconstitution ratio decreased the $t_{1/2}$ of reconstituted thylakoids 54% relative to the control thylakoids. The decrease in $t_{1/2}$ is dependent on the relative amount of LHC-II as shown in Table I where a 1:1 reconstitution decreased the $t_{1/2}$ by 25% relative to the control.

Another method of assessing the function of antenna Chl in the thylakoid is to measure electron transport activity induced by light preferentially absorbed by either Chl *b* or Chl *a*. The PSII reaction center contains predominantly Chl *a*, while the LHC-II contains approximately equivalent amounts of Chl *a*

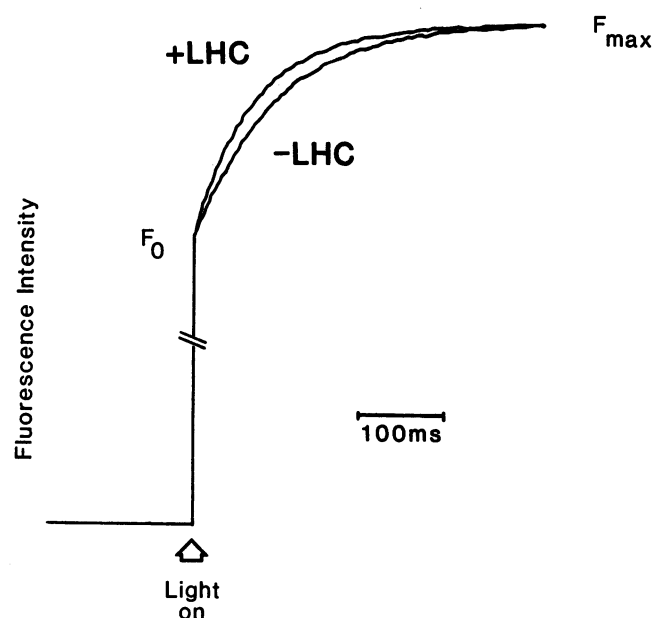


FIG. 4. Room temperature Chl fluorescence transients from control (-LHC) and reconstituted (+LHC) thylakoid membranes. F_0 and F_{max} are indicated. For ease of comparison the two transients are drawn with equal F_0 . Normally, the reconstituted sample (+LHC) would have a much higher F_0 . However, for both samples total variable fluorescence ($F_{max} - F_0$) is equal.

Table I. Half-Time of Room Temperature Chl Fluorescence Transients in the Presence of DCMU

Values in parentheses are % decrease in half-rise time as compared to the control.

	Reconstitution Treatment		
	Dialysis	Freeze-thaw	
LHC-II:thylakoids (Chl:Chl)	2:1	2:1	1:1
Thylakoid control (no LHC-II)	48.2 ± 1.5 ms	35.4 ± 1.9 ms	35.4 ± 1.9 ms
Reconstituted	37.8 ± 1.9 ms (22%)	16.2 ± 2.6 ms (54%)	26.6 ± 1.5 ms (25%)
Unreconstituted mixture	47.4 ± 5.5 ms	33.2 ± 5.7 ms	36.4 ± 5.3 ms

Table II. Electron Transport (DPC to DCPIP) Induced by 650 or 684 nm Light in Freeze-Thaw Reconstituted Samples

LHC:thylakoid	650 nm	685 nm	650/684
0:1 (thylakoid control)	8.9 ± 1.4^a	18.9 ± 1.3^a	0.47
1:1	12.8 ± 0.51	15.3 ± 0.4	0.84
2:1	14.3 ± 0.8	15.7 ± 1.2	0.91

^a $\mu\text{mol DCPIP (mg Chl)}^{-1} (\text{h})^{-1}$.

and Chl *b* (12). Thus, PSII reaction centers with functional LHC-II will catalyze more electron transport when exposed to 650 nm light (preferentially absorbed by Chl *b*) than will reaction centers without LHC-II.

Measurements of electron transport in reconstituted and control membranes in the presence of nonsaturating intensities of 650 and 684 nm light are shown in Table II. The ratio of electron transport in light preferentially absorbed by Chl *b* (650 nm) to that in light preferentially absorbed by Chl *a* (684 nm) increased with reconstitution of the LHC-II. This indicates that the reconstituted LHC-II is functionally associated with the PSII core

complex. Simply mixing LHC-II with thylakoids in equivalent amounts immediately prior to assay did not increase this ratio from the control membrane value (data not shown). Electron transport rates induced by saturating levels of light were identical in the control and reconstituted thylakoids (data not shown).

To test whether LHC-II was physically inserted into the thylakoid membranes upon reconstitution, density gradient centrifugation was carried out with all the samples. Thylakoids isolated from IML-treated barley reached a density equilibrium at 1.131 g/cm³ while aggregated LHC-II equilibrated at 1.171 g/cm³ (Fig. 5A). Reconstitution treatment of the LHC and thylakoid mixture produced a single broad Chl containing band (Fig. 5B) with an equilibrium density equal to that of the thylakoids in Fig. 5A. Although the band was not symmetrical across the gradient, it was homogeneous in composition when analyzed by absorption spectra, *i.e.* the Chl *a*:Chl *b* ratio was constant in all fractions of the peak. The broad distribution pattern may be the result of the formation of heterogeneously sized vesicles upon reconstitution treatment. When thylakoids and LHC-II were mixed without a freeze-thaw treatment and immediately loaded on the sucrose density gradient no apparent reconstitution was observed, as indicated by the separation of the sample into two Chl containing bands (Fig. 5B). Spectral analysis of the samples recovered in the upper and lower portions of the gradient showed no differences from the IML-treated thylakoids or the LHC-II run separately in Figure 5A.

Chl fluorescence emission spectra at 77°K of the freeze-thaw

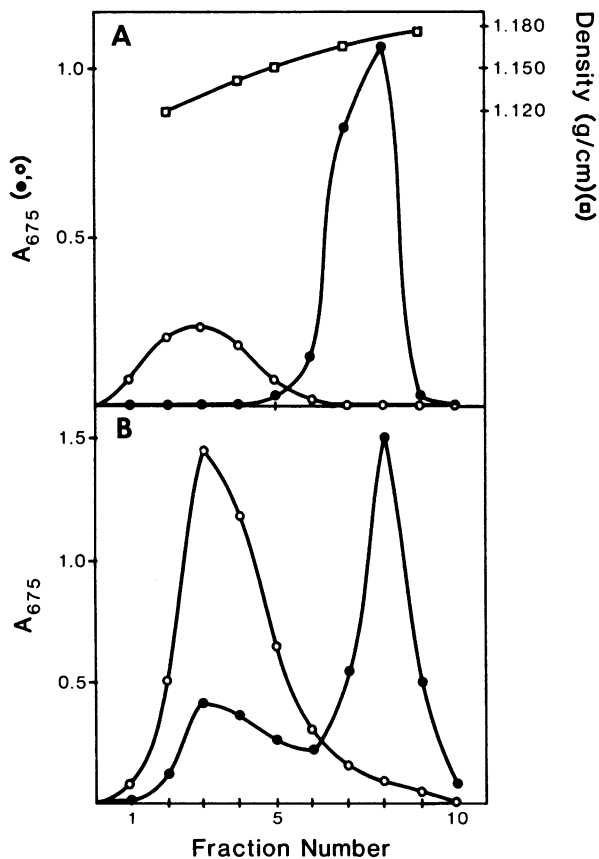


FIG. 5. Separation of Chl-containing fractions by density gradient centrifugation. A₆₇₅ was measured of each 20-drop fraction. A, (○), Thylakoid membrane control (25 μg Chl); (●), LHC-II control (50 μg Chl); (□), solution density of the fractions from the gradient. B, (○), Freeze-thaw reconstituted mixture 2:1 of LHC-II and thylakoids (75 μg Chl); (●), unreconstituted mixture of LHC-II (50 μg Chl) and thylakoids (25 μg Chl).

reconstituted samples are shown in Figure 6. Thylakoids from IML-treated seedlings exhibit fluorescence spectra that differ from that exhibited by fully developed thylakoids (*cf.* Fig. 3A with Fig. 6A) in that there is a general reduction of long wavelength emission, as discussed in more detail in Mullet *et al.* (25). Spectra from control LHC-II samples that were diluted with buffer equivalent to the volume of the thylakoids and put through the reconstitution treatment are shown in Figure 6B. The 700 and 681 nm emissions are nearly equal. Before the dilution and freeze-thaw treatment, the fluorescence spectrum of this sample resembled that in Figure 3C with a relatively lower 700 nm emission. The increase in the 700 nm component indicates that the LHC-II is aggregating in response to conditions simulating reconstitution. The fluorescence spectrum of the thylakoids reconstituted with LHC-II is shown in Figure 6C. The emission maximum has shifted from 685 nm in the thylakoids to approximately 684 nm. The presence of a short wavelength shoulder on this emission maximum is evidence that some of the reconstituted LHC-II remains at least partially disconnected from PSII, and retains its original 681 nm emission (Fig. 3C). However, the lack of a large 700 nm component in these spectra indicates that the LHC-II is not self-aggregating during reconstitution.

Greening of Thylakoid Membranes. When IML-treated barley

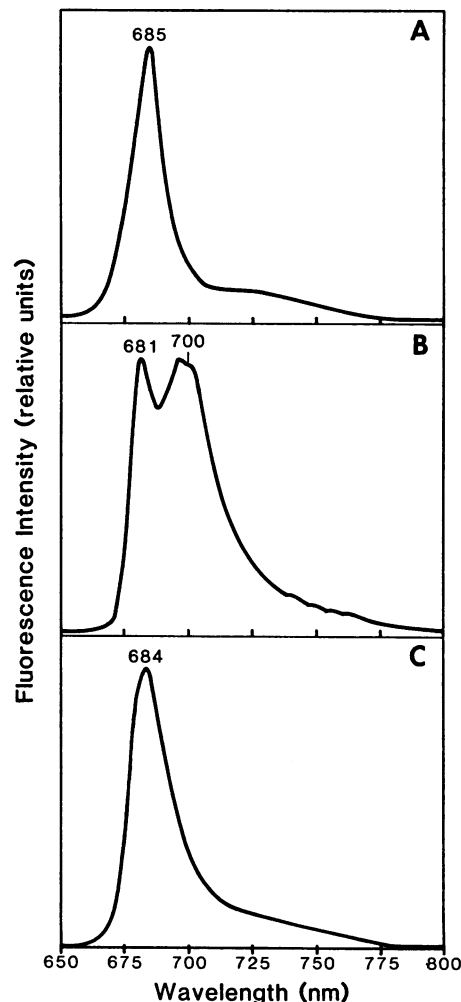


FIG. 6. Chl fluorescence emission spectra at 77°K of reconstituted samples. A, Control thylakoid membranes from IML-treated seedlings diluted and freeze-thawed in the absence of LHC-II; B, control LHC-II diluted and freeze-thawed in the absence of membranes; C, freeze-thaw reconstituted mixture of LHC-II and thylakoid membranes, 2:1, (Chl/Chl).

seedlings are placed in continuous illumination, Chl biosynthesis is stimulated, and functional LHC-II accumulates in the thylakoid membranes (1, 2). This *in vivo* development of LHC-II provides a natural control with which to compare the reconstitution system. Table III shows the $t_{1/2}$ of Chl fluorescence transients and the ratios of 650:684 nm light induced electron transport rates over the course of 24 h of greening. The data for fluorescence half-rise times for developing membranes (Table III) can be compared with the values for reconstituted membranes (Table I). The optimal reconstitution technique (freeze-thaw in a 2:1 LHC-II:thylakoid ratio) gave an equivalent percent decrease as that expected at about 16 h of greening *in vivo*. Comparing the electron transport data for the same preparation in Table II with that in Table III indicates that the 2:1 reconstitution gave a ratio of 650/685 induced electron transport expected for membranes between 12 and 24 h of greening *in vivo*. However, the Chl *a*:Chl *b* ratio in the reconstituted thylakoids was 1.85, much lower than the greened thylakoids at the 12 or 24 h time point. Assuming that all the Chl *b* in the greening membrane is bound to LHC-II and using Chl *a*:Chl *b* ratios for LHC-II and thylakoids from IML-treated seedlings as 1.15 and 12, respectively, the ratio of LHC-II Chl:thylakoid Chl after 18 h of greening (assumed Chl *a*:Chl *b* = 3.39) would be 0.64:1 (calculations derived from the data of Table III). This ratio of LHC-II Chl to the remaining thylakoid Chl in the greening membranes is 3.1 times less than the 2:1 ratio used for reconstitution. Thus, the efficiency of LHC-II sensitization of PSII core complexes in the reconstituted samples is less than that which occurs under natural greening conditions.

DISCUSSION

We report here the characterization of a reconstitution method which mimics the assembly of the chloroplast thylakoid membrane *in vivo*. This attempt has required two stages of research: the development of an LHC-II preparation that can be easily depleted of detergent, and the selection of a reconstitution system for adding this complex to incompletely developed membranes in which the function of LHC-II could be assessed.

We have isolated LHC-II from barley thylakoid membranes using a dialyzable detergent, OPOE. The preparation contains three polypeptides of 27, 25, and 24 kD. In addition, a 22 kD polypeptide was present in these preparations in somewhat variable amounts. This polypeptide has not previously been reported as a constituent of LHC-II. We believe that this protein is one of the polypeptides of LHC-I, the antenna of PSI (16, 24) based upon immunochemical reactivity using monoclonal antibodies in western blots (research in progress). The presence of this polypeptide in the LHC-II preparation may indicate a structural association between LHC-I and some of the LHC-II in the membrane. This polypeptide has no apparent effect on the reconstitution properties of the complex, since the relative

amount of the 22 kD protein in different preparations had no effect on reconstituted activity.

Using the OPOE preparation of LHC-II we have compared two reconstitution methods: dialysis and freeze-thaw. The dialysis-induced reconstitution method yielded membranes in which the LHC-II had less functional interaction with the PSII core complex than the freeze-thaw method. We believe this is due to less efficient insertion of the LHC-II into the thylakoid membrane. Ryrie *et al.* (29) also found that the insertion of purified LHC-II into liposome membranes was less efficiently driven by dialysis than by freeze thaw.

By comparing the light-harvesting properties of the freeze-thaw reconstituted preparations with the membranes developing *in vivo*, we have determined that about three times more LHC-II must be reconstituted *in vitro* to achieve activity equal to that in the samples greened for approximately 18 h in continuous light. If our reconstitution technique is randomizing the orientation of LHC-II insertion, then we would expect to need two times more LHC-II in the membranes to equal the *in vivo* activity. The apparent requirement for even more LHC suggests that some factors necessary for the interaction of LHC-II and PSII may be limiting in the IML-treated membrane. The membranes have an incompletely developed composition of lipids (18). For example, the $\Delta 3$ -*trans*-hexadecenoic fatty acid, which may play a role in the structure of LHC-II (8, 20), is present at reduced levels in IML-treated thylakoids compared to fully developed thylakoids. Alternatively, other factors such as linker polypeptides which may allow the recognition of PSII by LHC-II may be absent or limiting in the membrane. This would allow promiscuous association of LHC-II with other proteins in the thylakoid membrane, and prevent its efficient function in the transport of exciton energy to PSII. There is some precedent for a requirement for specific proteins to mediate the association of antenna pigment-protein complexes; in cyanobacteria, phycobilisome assembly is mediated by unpigmented, linker polypeptides of 27 to 33 kD (10). There is no evidence for the existence of a similar LHC-II/PSII linker protein at present; however, this reconstitution system would now allow the search for such a component.

In the study of photosynthetic membrane assembly using reconstitution systems, it should be born in mind that thorough dispersal of the aggregated LHC-II is a prerequisite to studying their subsequent binding to other membrane complexes. We wish to emphasize the special nature of the OPOE detergent in this regard. Several other research groups have reconstituted LHC-II prepared with Triton X-100 into artificial or thylakoid membranes (7, 21, 23, 29). This detergent is problematic for reconstitution studies because its low critical micelle concentration (0.3 mM) makes it difficult to remove from solution. The dialysis time required is too long to be practical for reconstitution. Instead, Triton is usually removed from the solution by absorp-

Table III. Greening of IML-Treated Seedlings under Continuous Illumination

Time of Continuous Illumination	Chl <i>a/b</i> ratio	Fluorescence Induction $t_{1/2}$ (% decrease)	DCPIP Reduction 650/684
<i>h</i>			
0	12.45 ± 2.0 ^a	30.2 ms	0.48 ^b
4	5.63 ± 0.37 ^a	22.8 (25%)	0.51
8	3.60 ^c	16.5 (45%)	0.54
12	3.55 ^c	14.8 (51%)	0.80
24	3.23 ^c	12.0 (60%)	0.93
Fully greened ^d	3.13 ^c	11.5 (62%)	0.94

^a Measured by method of Boardman & Thorne (4).
of MacKinney (19).

^d Greenhouse grown barley.

^b Normalized to Table II.

^c Measured by method

tion to Biobeads SM-2 (7, 21, 29). Abrupt removal of the detergent in this way causes gross aggregation of the protein, which apparently is not easily reversed. Sprague *et al.* (31) have reported that prior aggregation of LHC-II causes hexagonal lattice crystal-like clusters of LHC-II to appear in freeze-fractured reconstituted membranes. These lattices have been observed in all reports where aggregated LHC-II was reconstituted into artificial membranes. They were also observed when Day *et al.* (7) reconstituted LHC-II into thylakoid membranes isolated from IML-treated barley seedlings. We believe that the use of OPOE to separate aggregated LHC-II before reconstitution is an important improvement over the use of Triton X-100; the OPOE/LHC-II mixture can be added to thylakoids without residual detergent micelles causing a loss of functional activity of the membranes, as occurs with Triton X-100. This property of OPOE arises from the fact that its micelles can easily be removed from solution either by simple dilution or by dialysis due to its high critical micelle concentration.

LHC-II has been successfully reconstituted with PSII using other membranes besides thylakoids from IML-treated barley. Murphy *et al.* (26) reported reconstituting LHC-II with PSII particles in soybean phosphatidyl choline liposomes. However, their PSII preparation was derived from mature membranes and contained substantial quantities of LHC-II already bound to the PSII core complexes. Thus, the interaction they observed may not be the primary association between LHC-II and PSII, but rather a nonspecific aggregation of LHC-II into an already functioning pool of antenna. The problem of PSII preparations that already contain LHC-II was solved by Larkum and Anderson (17). They used a highly purified PSII preparation for reconstitution with LHC-II and PSI in liposomes prepared from chloroplast diacyl lipids. However, the amount of purified PSII available in their studies was limiting so that their analysis of functional interactions of LHC-II and PSII core complexes was restricted to fluorescence emission and excitation spectra. The use of intermittent-light treatment to prepare membranes for reconstitution circumvents both of the above difficulties. Thylakoids from IML-treated seedlings contain no functional LHC-II and only low levels of the apoproteins. Furthermore, the quantity of membranes available from a single preparation is not limiting in most cases. An additional benefit from using these thylakoid membranes for reconstitution is that the membranes preserve many of the normal lipid and intrinsic protein characteristics of the chloroplast thylakoid.

In summary, we have demonstrated that OPOE solubilized LHC-II can functionally integrate into thylakoid membranes so that a portion of it sensitizes PSII photochemistry. We can not explain why the *in vitro* system requires more LHC-II integration than normal membrane development to achieve an equal level of PSII sensitization. The reason does not appear to involve LHC-II self-aggregation (lack of the 700 nm emission, a diagnostic indicator of aggregation, in reconstituted membranes; Fig. 6). It therefore seems likely that the LHC-II becomes randomly distributed in reconstituted thylakoids, a phenomenon which apparently does not occur *in vivo*. This study suggests to us that there may be a 'linker component' which regulates proper association of the LHC-II with PSII core complexes during membrane development.

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