Crystallization of the Light-harvesting Chlorophyll $a/b$ Complex within Thylakoid Membranes

MARY K. LYON and KENNETH R. MILLER
Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

ABSTRACT We have found that treatment of the photosynthetic membranes of green plants, or thylakoids, with the nonionic detergent Triton X-114 at a 10:1 ratio has three effects: (a) photosystem I and coupling factor are solubilized, so that the membranes retain only photosystem II (PS II) and its associated light-harvesting apparatus (LHC-II); (b) LHC-II is crystallized, and so is removed from its normal association with PS II; and (c) LHC-II crystallization causes a characteristic red shift in the 77°K fluorescence from LHC-II. Treatment of thylakoids with the same detergent at a 20:1 ratio results in an equivalent loss of photosystem I and coupling factor, with LHC-II and PS II being retained by the membranes. However, no LHC-II crystals are formed, nor is there a shift in fluorescence. Thus, isolation of a membrane protein is not required for its crystallization, but the conditions of detergent treatment are critical. Membranes with crystallized LHC-II retain tetrameric particles on their surface but have no recognizable stromal fracture face. We have proposed a model to explain these results: LHC-II is normally found within the stromal half of the membrane bilayer and is reoriented during the crystallization process. This reorientation causes the specific fluorescence changes associated with crystallization. Tetrameric particles, which are not changed in any way by the crystallization process, do not consist of LHC-II complexes. PS II appears to be the only other major complex retained by these membranes, which suggests that the tetramers consist of PS II.

A number of integral membrane proteins have been crystallized in two dimensions in recent years, yielding detailed information about the exact structure of these proteins (1–6). One of the proteins that is amenable to crystallization is the light-harvesting complex (LHC-II) found in the photosynthetic membranes of chloroplasts from green plants. After its removal from the membrane by detergent solubilization, large LHC-II crystals are formed, and both two- and three-dimensional reconstructions of the complex have been reported (7–9). Three-dimensional reconstruction has shown that individual LHC-II complexes are highly asymmetric. A large portion of each complex is exposed on one side of the crystal sheet, whereas only a small portion of the complex is exposed on the opposite side of the crystal sheet (9).

Although crystallization of an isolated protein yields information on the protein's structure, it is not a technique that gives information on the position within the native membrane. Photosynthetic membranes, or thylakoids, are sac-like structures which are aligned, or stacked, on specific areas of their outer surface. They contain at least 43 different polypeptides (10). Freeze-fracture and fractionation studies have shown that thylakoid proteins are not uniformly distributed in stacked and nonstacked regions (11–14). Thus, thylakoids are complex, both in terms of their structural arrangement and in the number of components found within the membrane. Because of this complexity, there is disagreement in the literature concerning the exact position of LHC-II in the membranes (15–18).

In this paper, we describe crystallization of LHC-II within thylakoids by a mild detergent treatment. The conditions required to form LHC-II crystals yield information on the crystallization process itself, whereas the specific changes found in membranes with crystallized LHC-II yield information on the normal position of LHC-II in thylakoids.
Materials and Methods

Detergent Treatment: Pea seedlings (Pisum sativum, Alaskan variety) were grown in vermiculite for 3 wk before they were harvested, and thylakoids were isolated as described (11). Several detergents, including Tritons X-45, X-100, and X-114 were tested for their ability to produce lattices of LHC-II. The most extensive lattices were produced by Triton X-114, and further experiments were then conducted exclusively with that detergent.

The isolation buffer was 150 mM sucrose, 5 mM MgCl2, and 50 mM Tricine, pH 7.5. Thylakoids were resuspended in 15 mM NaCl, 5 mM MgCl2, 20 mM HEPES, pH 7.5, centrifuged at 12,000 g for 10 min, and resuspended in 15 mM NaCl, 50 mM sucrose, 5 mM MgCl2, 20 mM HEPES, pH 7.5, to a chlorophyll concentration of 0.20 mg/ml. 20% (wt/vol) Triton X-114 (Sigma Chemical Co., St. Louis, MO) in the same buffer was added to give the detergent-to-chlorophyll ratio of 10:1 indicated in the Results section. The mixture was stirred for 30 min, then was centrifuged at 47,800 g for 10 min. The membranes were resuspended in a large volume of 15 mM NaCl, 50 mM sucrose, 20 mM HEPES, pH 7.5, and centrifuged at 12,000 g for 30 min. All procedures were carried out at 4°C. Chlorophyll concentrations were determined by the method of Arnon (19).

Freeze-etch and Freeze-fracture Procedures: The same samples were used for both freeze-fracturing and freeze-etching. Glycerol infiltration was avoided to ensure that freeze-etched and freeze-fractured samples were directly comparable. Freshly prepared samples were resuspended in isolation buffer and left on ice for at least one-half hour to be certain that all membranes were in a stacked configuration. They were centrifuged at 12,000 g for 20 min, resuspended in 10 mM MgCl2, centrifuged at 12,000 g for 20 min, and frozen by immersion in FREON 22 (DuPont Co., Wilmington, DE). For etching procedures, samples were sampled at −100°C and etched at 5 min at the same temperature. For fracturing procedures, the samples were fractured at −110°C. Both freeze-fracturing and freeze-etching were done in a Balzers 400 freeze-etch device (Balzers, Hudson, NH). Micrographs were taken on a JEOL 100B or Philips 410 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Gel Electrophoresis: Samples were run on a Laemmli gel system (20). The sample buffer consisted of 2% SDS, 0.05 M Na2CO3, 0.05 M dithiothreitol, 12% sucrose, and 0.04% bromphenol blue. The resolving gel was 15% acrylamide, with a 4% stacking gel. Electrophoresis was done at 4°C.

Fluorescence Spectra: Low temperature fluorescence emission spectra were obtained as described by Biggins (21). The excitation light was 435 nm. 2',7'-Dichlorofluorescein (Eastman Laboratory and Specialty Chemicals, Rochester, NY) was used as a fluorescent standard at a concentration of 2 × 10−3 M.

Optical Diffraction: Optical diffraction patterns were recorded on a bench constructed with a 1200-mm focal length after the design of Salmon and DeRosier (22), and illuminated with a 5-mW helium laser.

Results

Three different photosynthetic membrane preparations were used for this study. Control samples were isolated according to standard techniques and never subjected to detergent extraction. Detergent-treated membranes were extracted with the nonionic detergent Triton X-114 at a detergent/chlorophyll ratio of 10:1, or with the same detergent at a 20:1 ratio. Each sample was then examined biochemically (by gel electrophoresis), structurally (by freeze-fracturing and freeze-etching), and for excitation energy distribution (by low-temperature fluorescence).

Membranes that had been extracted with Triton X-114 at a ratio of 10:1 showed a series of dramatic structural changes, as illustrated in Fig. 1, a–c. Extensive lattices of particles were observed on the surfaces of 10:1-extracted membranes (Fig. 1, a and b). At high magnifications, the structure of these lattices could be observed (Fig. 1 c). Distinct depressions were arrayed hexagonally, thus forming parallel, staggered rows. Small particles made up rows between the depressions (Fig. 1 c). When the micrograph is viewed at an angle, it can be seen that some of these particles extended into the rows of depressions.

Large, tetrameric particles were also found on the surfaces of the 10:1 Triton X-114–extracted membranes (Fig. 1, a–c). Tetrameric particles were found on the inner surfaces of the stacked regions of thylakoids that had not been detergent extracted (Fig. 1, d–e and references 11–13). The tetramers found on the 10:1-extracted membranes (Fig. 1 c) appeared identical to those found on control membranes (Fig. 1 e). However, the distribution of these particles had changed with detergent treatment. In the detergent-extracted sample, the tetrameric particles were interspersed between lattice regions (Fig. 1, a and b), whereas they were evenly distributed over the membrane surface in control thylakoids (Fig. 1 d).

The lattice structures were also visible in freeze-fracture, as shown in Fig. 2a, which indicates that they extended through the membrane interior. Control membranes displayed well-defined inner (E) and outer (P) fracture faces (Fig. 2, b and c). The stacked and nonstacked regions of each fracture face were also distinguishable in the control membranes. In contrast, it was often difficult, even in nonlattice regions, to distinguish the E and P faces of the 10:1–extracted membranes. However, the presence of both large particles (arrowheads, Fig. 2a) and small particles (thin arrows, Fig. 2a) near or within areas of lattice demonstrated that both halves of the membrane bilayer were involved in lattice formation. Large particles, normally found in the inner fracture face in stacked regions (EFS), were found in clusters between the areas of lattice. In contrast, smaller particles, normally found on the outside fracture face in stacked regions (PFs), were frequently interspersed within the lattices. No areas similar to the inner or outer fracture face of nonstacked regions of control membranes (Fig. 2, b and c) were observed in the 10:1 Triton-extracted samples.

The structural characteristics of the lattices found in the 10:1 sample matched those reported for two-dimensional crystals of purified LHC-II (7–9). Optical diffraction patterns taken through the lattice regions (inset, Fig. 2a) show the hexagonal pattern and 12.5-nm spacing characteristic of purified LHC-II crystals (7). Individual particles within the crystal were ~90 Å in diameter.

Photosynthetic membranes treated with a higher concentration of detergent (20:1) differed from both 10:1 samples and control membranes. The surface of the 20:1-extracted membranes (Fig. 3a) was very similar in appearance to the
surface of control membranes (Fig. 1 d). Distinct tetrameric particles were clearly visible, though they were spaced further apart than in the control membranes. However, freeze-fractured preparations of the 20:1 sample (Fig. 3 b) were dramatically different in appearance than were freeze-fractured control membranes (Fig. 2, b and c). As with the 10:1-extracted membranes, it was difficult to distinguish clearly E and P faces. Small particles (thin arrow, Fig. 3 b) were found next to large particles (thick arrow, Fig. 3 b), something not observed in control membranes. There appeared to be fewer particles per unit area than normally found in the stacked regions of control membranes. No lattices were observed in the 20:1-extracted membranes. Thus, whereas the surfaces of the 20:1 detergent-treated membranes were essentially normal in appearance, the fracture faces showed that considerable rearrangement of the membrane had occurred.

Gel electrophoresis was used to determine the polypeptide components of each sample (Fig. 4). The 10:1- and 20:1-extracted membranes retained the same polypeptides (lanes 2 and 3, Fig. 4). LHC-II consists of two heavy bands and at least one minor band at ~29–26 kD (23–26), each of which was retained in both the 10:1 and 20:1 samples (Fig. 4). Additional polypeptides were also retained. On the basis of molecular weight, many of these polypeptides can be assigned to photosystem II (PS II) (27–31). The Triton-treated membranes have retained polypeptides at ~56 and 48 kD. These polypeptides have been associated with PS II reaction centers (27–31). Additional polypeptides that are associated with PS
**FIGURE 3** (a) Freeze-etched membranes from the 20:1 sample. Tetrameric particles are interspersed over a smooth matrix. No lattice structures were found in these samples. Bar, 0.19 μm. × 106,400. (b) Freeze-fractured membranes from the 20:1 sample. Although both large particles (arrowhead) and small particles (thin arrow) are visible, it is impossible to distinguish clearly E and P faces. Bar, 0.1 μm. × 100,500.

**FIGURE 4** Gel electrophoresis of whole membranes (lane 1), 10:1 sample (lane 2), and 20:1 sample (lane 3). The light-harvesting complex (LHC-II) is retained by all samples. The 10:1 and 20:1 samples also retained other polypeptides. CF₁ (coupling factor, alpha- and beta-subunits); PS I (photosystem I apoprotein and chlorophyll-binding polypeptide); PS II-RC (photosystem II, reaction center polypeptides); PS II-O₂ (photosystem II, polypeptides associated with oxygen evolution).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chlorophyll a/b ratio</th>
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<tr>
<td>Control</td>
<td>2.82 ± 0.21</td>
</tr>
<tr>
<td>10:1</td>
<td>1.94 ± 0.13</td>
</tr>
<tr>
<td>20:1</td>
<td>1.74 ± 0.03</td>
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Ratios are based on a minimum of three experiments.

II oxygen evolution have also been retained (29-31). Polypeptides at ~110 and 68 kD have been solubilized by Triton X-114 and removed from the membranes. Polypeptides with these molecular weights are associated with photosystem I (PS I) (32). Two additional bands, at ~66 kD, have also been removed from the membranes. These two bands are the alpha- and beta-subunits of the coupling factor (10, 26). Several other polypeptides, of unknown origin, have also been solubilized. However, the 10:1 and 20:1 samples did not differ from each other significantly in their polypeptide pattern, and both retained multiple polypeptides in addition to LHC-II.

Almost all of the chlorophyll b found in thylakoids is bound by LHC-II (33), with only a minor amount of chlorophyll b found in the light-harvesting complex associated with PS I (33). Therefore, we have determined the chlorophyll a/b ratio of each membrane preparation to obtain a more quantitative estimate of the relative amounts of LHC-II in each of the three membrane preparations (Table I). The chlorophyll a/b ratios confirmed the results of gel electrophoresis, as detergent treatment had resulted in an increase in the relative amount of chlorophyll b, and thus of LHC-II. In addition, it was found that the membranes treated at a 20:1 ratio were somewhat more enriched for LHC-II than were the membranes treated at a 10:1 ratio.

Low temperature fluorescence spectra have been used in previous studies to examine the degree of energetic coupling among PS I, PS II, and LHC-II (34, 35). Specific fluorescent peaks have been ascribed to each complex: LHC-II, with a peak at 680-685 nm, PS II, with a peak at 695 nm, and PS I, with a peak at 735 nm (35). 77K fluorescence spectra of the detergent-treated membranes revealed two specific changes from control membranes (Figs. 5 and 6). As shown in Fig. 5, the amount of fluorescence from PS I was drastically reduced with respect to fluorescence from PS II. This confirms the
results of gel electrophoresis, which showed that PS I-related polypeptides were removed by detergent extraction. The second change in this sample involved fluorescence from LHC-II. As shown in Fig. 6, the distinct 685 nm peak from LHC-II both increased in size and shifted slightly to the red (~690 nm). This red-shifted peak in the 10:1 sample overlaps with the PS II peak. In contrast, distinct LHC-II and PS II peaks are observable in both control and 20:1 samples (Fig. 5 and 6). Thus, the red-shifted LHC-II fluorescence peak was found only with the membranes that contained extensive areas of LHC-II crystal.

DISCUSSION
Crystallization of a protein within a membrane provides two different types of information. First, it yields information on the crystallization process itself. Second, crystallization effectively removes a protein or protein complex from its normal structural relationship with other proteins in the membrane. As a consequence, crystallization can provide information on the structural relationship of two or more proteins within a membrane.

Identity of Lattices
We conclude that the large arrays found in the 10:1 sample are crystals of LHC-II. Their appearance in both freeze-etched and freeze-fractured samples is identical to the appearance of crystals of purified LHC-II (7-9). The optical diffraction pattern and center-to-center spacing is the same as in pure LHC-II crystals (7). Furthermore, the changes in the fluorescence spectrum are in agreement with changes in fluorescence of purified LHC-II after aggregation (23, 24). In both studies, it was found that uncrystallized, purified LHC-II gives a sharp emission peak at 680-681 nm. In contrast, crystallized LHC-II gives a broad emission peak, ranging from 680 to 695 nm (23, 24). A similar fluorescence change is found in the 10:1 sample, which has extensive areas of crystal (Fig. 1, a and b), but not in untreated membranes or the 20:1 sample, which has no crystals (Figs. 1d and 3,a and b). Thus, there is a correspondence between the appearance of LHC-II crystals and a broadening of the fluorescence emission peak from LHC-II.

Gel electrophoresis demonstrated that many polypeptides remained in the membranes after detergent treatment at a 10:1 ratio. Three of these polypeptides make up the light-harvesting complex (23-26). Based on molecular weight, almost all of the remaining polypeptides can be ascribed to the PS II reaction center (27, 28) or PS II oxygen evolution (29-31). Consequently, both the 10:1 and 20:1 samples consisted of membranes with two chlorophyll-protein complexes, PS II and LHC-II. The only apparent difference between the two samples was that LHC-II was crystallized in the 10:1 sample.

Crystallization Process
Crystallization of isolated membrane components is a relatively recent achievement (1-6). Crystals of cytochrome oxidase have been formed in mitochondrial membranes by Triton X-100 extraction in a manner similar to the procedure we have used to obtain LHC-II crystals in thylakoids (1). However, it has generally been assumed that detergent solubilization and purification of a protein or protein complex is required before crystallization can occur (36). From the present study, it is clear that this is not an absolute requirement. The areas of LHC-II crystal that are visible in detergent-treated membranes are virtually identical to crystals of purified LHC-II (7-9). Yet, the detergent-treated membranes with large areas of LHC-II crystal retain many additional polypeptides, as seen by gel electrophoresis. The membranes also
retain structures found in untreated thylakoids, notably the large particles found on the EFs face and the tetrameric particles found on the inner membrane surface in stacked regions. Consequently, the LHC-II crystals found in the 10:1 sample have been formed in situ without extraction of the LHC-II complex from the membrane and in the presence of other polypeptides.

Although purification may not be required for crystallization, it is likely that a high concentration of the proteins to be crystallized is required. LHC-II accounts for ~50% of the protein content of thylakoids (33), and our detergent treatment has increased that percentage, as revealed by both gel electrophoresis and changes in the chlorophyll a/b ratios. Crystallization of a pore protein complex within mitochondrial outer membranes has been reported recently (2). The pore protein complex accounts for 68–80% of the protein mass in these membranes (2). It is believed that crystallization occurs as a result of the loss of phospholipids, with subsequent enrichment of the protein complexes within the mitochondrial membrane (2). It appears that intramembranous crystallization of cytochrome oxidase (1), the mitochondrial pore, and LHC-II, occurs by similar processes involving enrichment of the lipid bilayer for a single protein complex.

However, a high concentration of protein cannot be the only requirement for crystallization. The membranes treated with Triton X-114 at a 20:1 ratio also retained LHC-II and were in fact more enriched for LHC-II than was the 10:1 sample, as judged by the chlorophyll a/b ratios, but no crystalline arrays were found in the 20:1 membranes. Detergent concentration has been found to be critical for crystal formation with purified LHC-II, as excess detergent produces inferior crystals (8). In the present study, an increase in the detergent concentration also prevented crystal formation.

Detergents or other amphiphilic molecules are believed to play a critical role in crystal formation by coating the hydrophobic portions of membrane proteins (36). Small patches of LHC-II crystal have been found in thylakoids that had been enriched in lipid content by addition of phosphatidyl glycerol (37). This suggests that detergent treatment may work by a similar mechanism, with detergent molecules being inserted into the membrane, rather than coating the surface. This mechanism is in agreement with the concept that amphiphilic molecules coat the hydrophobic portions of proteins during crystallization (36).

Triton X-114 has had an additional effect on LHC-II. Studies on crystals of purified LHC-II have shown that the individual complexes are arranged head-to-tail (7, 9). As a consequence, freeze-fracture through LHC-II crystals produces two identical fracture faces (8). However, LHC-II is unidirectionally positioned in control thylakoids (38, 39). The unidirectional position of LHC-II in whole thylakoids has been confirmed by proteolysis effects on LHC-II in the native membrane and by agglutination of various membrane preparations with antibodies directed against LHC-II (38, 39). Therefore, formation of LHC-II crystals in the Triton X-114-treated membranes could only have occurred if some of the LHC-II complexes had been reoriented in the membrane.

**Structural Relationship of LHC-II and PS II**

Crystallization of LHC-II removes it from its normal structural relationship with other components in the 10:1 sample. Therefore, an analysis of the effects of crystallization of LHC-II in a membrane that retains PS II provides specific information on the normal structural relationship of LHC-II and PS II.

One of the distinct features of thylakoids treated at a 10:1 detergent/chlorophyll ratio was the appearance of tetrameric particles on the membrane surface (Fig. 1c). The retention of tetrameric particles in the 10:1 sample after formation of LHC-II was accompanied by a retention of the large E face particles (Fig. 2a). Both the tetramers and the large EFs particles were localized between patches of LHC-II crystals. This suggests that the EFs particle observed in freeze-fracture preparations is associated with the tetrameric particle found on the membrane surface in etched preparations. The same suggestion has been made in previous studies (12, 40).

Several previous studies have suggested that LHC-II is associated with PFs. Purified LHC-II, when incorporated into liposomes, forms particles of ~80 Å (25), which is nearly the size of particles found on the PFs face (12, 17, 41). A mutant barley plant, which lacks the ability to synthesize chlorophyll b, has been found to have almost no LHC-II in the thylakoids (17). Such a mutation resulted in a change only on the PFs face (17). Ultrastructural studies on state I-, state II-associated phosphorylation, which affects LHC-II, have demonstrated changes only on the P face (42). All of these results suggest that LHC-II is associated with the small particles found on the PFs face.

There is, however, conflicting evidence concerning LHC-II localization. There is some evidence that the large particle on the EFs face may consist of PS II reaction centers with associated LHC-II. In particular, PS II mutants, which lack PS II reaction centers, lack the EFs particle (43, 44) and the tetrameric particle found on the E surface (43). The association of LHC-II with the EFs particle has been deduced primarily from studies on peas grown under intermittent light (15). From the results of these studies, it has been suggested that incorporation of LHC-II into thylakoids results in an increase in the average size of EFs particles, and that four LHC-II complexes are associated with each PS II reaction center (15). A second chlorophyll b barley mutant has been found to have a reduced number of particles on the P face, a slight reduction in the size of EFs particles, and a complete reorganization of the structure of the tetrameric particles (41). Last, it has been suggested that LHC-II may vary in its localization on freeze-fracture samples, since it is found on the P face under specific conditions and on the E face under other conditions (45).

As a result of these conflicts in the literature, several models for the organization of LHC-II and PS II have been proposed. It has been suggested that four LHC-II complexes are associated with each PS II reaction center (14–16, 41). It has also been suggested that LHC-II and PS II fractionate to the same plane, giving rise to the EFs particle, and that the tetrameric particle derives its four-particle appearance from four LHC-II complexes (15, 16, 41). A second model that has been proposed differs significantly. In this model, several LHC-II complexes are associated with each PS II reaction center, but the freeze-fracture plane separates the LHC-II and PS II complexes, so that LHC-II is found on the P face and PS II is found on the E face (17). It has also been suggested that LHC-II may partition to either fracture face (45). More recently, it has been proposed that there are two populations of LHC-II—one of which is mobile and can migrate to the stromal
lamellae, and a second population which is tightly bound to PS II (18, 42).

From the results of this study, it appears that LHC-II is not associated with the tetrameric particles but is associated with the P face. Crystallization of LHC-II has resulted in the loss of any recognizable areas of P face. Furthermore, particles within fractured LHC-II crystals are approximately the same diameter as particles found on the PFs. Our interpretation of the changes associated with LHC-II crystallization is schematically represented in Fig. 7. LHC-II is associated with each PS II reaction center, but the two complexes partition separately during freeze-fracture. During crystallization, some of the LHC-II complexes are inverted. These changes result in a complete change in the appearance of the fracture faces and give rise to the changes in the fluorescence emission spectra from LHC-II. Tetrameric and EFs particles exist side by side with LHC-II crystals and are not changed in any way by the crystallization process. Observations on the alignment of all particles on both EFs and PFs faces and inner and outer membrane surfaces in stacked regions also support this model (13).

However, the possibility that there are two populations of LHC-II (18, 42) cannot be ruled out. If this were the case, the crystals observed here might have been formed from the mobile population of LHC-II, whereas the tightly bound population could have remained associated with the tetramers observed on the E surface.

The ability to form crystals of a single component within a membrane provides a unique opportunity to study structural relationships within that membrane. However, it must be noted that crystallization of isolated components is required before studies of relatively intact membranes can provide any information. For example, if LHC-II had not been purified and crystallized (7–9), we could not have identified the components of the crystals in this study, nor would we have been able to draw any conclusions regarding the structural relationships of LHC-II and PS II.

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