Revisiting the Supramolecular Organization of Photosystem II in Chlamydomonas reinhardtii

Background: Light-harvesting complex II (LHCII) proteins associate with photosystem II (PSII) to form a supercomplex.

Results: We isolated an active PSII supercomplex from thylakoids solubilized with dodecyl-α-d-maltoside and visualized it in a large projection map with an electron microscope.

Conclusion: The novel PSII supercomplex harbored three LHCII trimers on each side.

Significance: The large and active PSII-LHCII supercomplex is engaged in green algal photosynthesis.

Photosystem II (PSII) is a multiprotein complex that splits water and initiates electron transfer in photosynthesis. The central part of PSII, the PSII core, is surrounded by light-harvesting complex II proteins (LHCII). In higher plants, two or three LHCII trimers are seen on each side of the PSII core whereas only one is seen in the corresponding positions in Chlamydomonas reinhardtii, probably due to the absence of CP24, a minor monomeric LHCII. Here, we re-examined the supramolecular organization of the C. reinhardtii PSII-LHCII supercomplex by determining the effect of different solubilizing detergents. When we solubilized the thylakoid membranes with n-dodecyl-β-d-maltoside (β-DM) or n-dodecyl-α-d-maltoside (α-DM) and subjected them to gel filtration, we observed a clear difference in molecular mass. The α-DM-solubilized PSII-LHCII supercomplex bound twice more LHCII than the β-DM-solubilized supercomplex and retained higher oxygen-evolving activity. Single-particle image analysis from electron micrographs of the α-DM-solubilized and negatively stained supercomplex revealed that the PSII-LHCII supercomplex had a novel supramolecular organization, with three LHCII trimers attached to each side of the core.

Conversion of solar energy to electrochemical energy in photosynthetic eukaryotes is conducted by large pigment–protein supercomplexes embedded in thylakoid membranes. Using light energy, photosystem II (PSII) splits water and reduces plastoquinone. PSII is made up of two parts: an oxygen-evolving/electrogenic core complex and a surrounding light-harvesting antenna complex (LHCII). Numerous attempts have been made to isolate and characterize PSII from a wide range of organisms, including higher plants, eukaryotic algae, and cyanobacteria (for review, see Ref. 1). Recently, atomic structures of the PSII core dimer isolated from cyanobacteria were determined at 3.5–1.9 Å resolution (2–4), and the crystal structures of LHCII were determined at 2.7–2.5 Å for the trimers (5, 6) and at 2.8 Å for the monomer (7). Structural information for the entire PSII-LHCII supercomplex, however, is limited.

Although its resolution is relatively low compared with crystallography, single-particle analysis of electron micrographs has revealed the two- and three-dimensional structures of the PSII-LHCII supercomplex (1, 8, 9). Several LHCII trimers are associated with the two sides of the dimeric PSII core, each of which consists of a few major LHCII trimers and a few minor LHClI monomers (1, 10); the trimers and the core are bordered by the monomers (1). When spinach thylakoid membranes are solubilized by n-dodecyl-β-d-maltoside (β-DM) (9, 11–13), one LHCII trimer binds strongly to each side of the core (C₆S₂PSII-LHCII supercomplex), but when they are solubilized by n-dodecyl-α-d-maltoside (α-DM), the PSII-LHCII supercomplexes organize as C₆S₂M₁₋₂L₀₋₁ or C₆S₂M₀L₁₋₂, where one to two moderately bound LHCII trimers and/or one loosely bound LHCII trimer, or one to two loosely bound LHClI trimers, are associated with the C₆S₂-type supercomplex (14). When Arabidopsis thaliana thylakoid membranes are solubilized with α-DM and fractionated by gel filtration (15) or sucrose density gradient (16), the C₆S₂M₀ organization is the largest type seen. These single-particle images of the detergent-solubilized PSII-LHCII supercomplexes were recently confirmed in the membranes by means of cryoelectron tomography (17, 18).

When the PSII-LHCII supercomplex from the green alga Chlamydomonas reinhardtii is prepared with a relatively high concentration of β-DM (50 mM; 2.6%), C₆S₂ organization is observed much as it is in higher plants (8), and when n-tridecyl-β-d-maltoside is used to purify the monomers and multimers of the supercomplex, no association of M- or L-trimmers with PSII is observed, either (19). We have tentatively attributed this to...
the absence of CP24 (20), a minor monomeric LHCII protein specific to higher plants that may serve as a linker between PSII core subunits and an M-trimer (21, 22). However, although α-DM is used to isolate PSII-LHCII supercomplexes with M-trimer(s) from higher plants, it has never been used for that purpose in green algae. It thus remains unclear whether the C. reinhardtii PSII-LHCII supercomplex contains M-trimer(s), and if not, whether the PSII-LHCII supercomplex organization differentiates higher plants from green algae. The association of L-trimer is also unclear. Its binding has not been observed in A. thaliana, and the PSII-LHCII supercomplex with a stoichiometric amount of L-trimers (the C₅S₂M₁L₂ PSII-LHCII supercomplex) has not even been confirmed in spinach, although its existence has been proposed (14).

Here we examined the effects of α- and β-DM on the solubilization of the thylakoid membranes from C. reinhardtii and demonstrated that the green algal PSII could indeed form the C₅S₂M₁L₂ supercomplex in the α-DM solubilized membranes. This largest PSII-LHCII supercomplex retained a high oxygen-evolving activity.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—C. reinhardtii WT strain 137c, Fus7 (ΔPsbA), and Fus6 (ΔPetD) were obtained from the Chlamydomonas Center. The PSI-less (ΔPsaA) mutant was a gift from Dr. Kevin Redding, Department of Chemistry and Biochemistry, Arizona State University (supplemental Fig. S1).

All the strains were grown in Tris acetate-phosphate medium at 10,000 rpm in low light (10–30 microeinsteins m⁻² s⁻¹). The thylakoid membranes were adjusted to 0.4 mg of chlorophyll (Chl)/ml in a 25 mM MES buffer, pH 6.5, and solubilized for 5 min on ice in the isolation buffer containing 25 mM MES, 1 M betaine, 10 mM MgCl₂, 10 mM CaCl₂, 5 mM EDTA, and 12.5% (w/v) glycerol, pH 6.5, and disrupted twice with the BioNeb disruption system (Glas-Col, LLC, IN) at 7.5 kgf/cm².

Isolation of Thylakoid Membranes—Thylakoid membranes were isolated from C. reinhardtii as reported previously (24, 25), with the following modifications. Cells were harvested at early to mid-log phase (2–5 x 10⁶ cells/ml), resuspended in the thylakoid preparation buffer with the indi- cated concentration of CaCl₂ in the presence of 2 mM potassium ferricyanide and 0.5 mM 2,6-dichloro-benzoquinone at 20 μg of Chl/ml. Relative oxygen-evolving activity per reaction center was calculated as the number of Chl molecules/reaction center in the respective complexes. The Chl numbers bound to PSII-LHCII supercomplexes were estimated based on earlier crystal studies (70 for the PSII core dimer (4), and 14 and 42 for an LHClI monomer and trimer, respectively (6)).

Electron Microscopy and Single-particle Analysis—The sucrose contained in the fraction of the PSII-LHCII supercomplex harvested by S/DG centrifugation was removed by dialysis in a 25 mM MES, pH 6.5, buffer containing 0.02% α-DM. The samples were then diluted 3-fold in the same buffer, applied to glow-discharged carbon-coated copper grids for 30 s, and negatively stained for 30 s three times with 2% uranyl acetate. Electron micrographs were taken with an H-7650 Zero-A electron microscope (Hitachi) at 80 kV and × 48,000 magnification. Catalase crystals were used as the internal standard for calibrating length information (26). Images free from astigmatism and drift were recorded on a CCD camera (1,024 x 1,024 pixels) at a pixel size of 3.6 Å and a underfocus value of 0.8 μm. Their resolution, estimated by the Fourier ring correlation method (27), was 16.8 Å. From 300 images, 5,560 particles were extracted in a 150 x 150-pixel area with X3DPreprocess software (28) and band-pass filtered between 1,000 and 5 Å with Bfilter from the Bsoft package (29, 30). Image analysis was carried out using SPIDER software (31, 32). Particles were aligned and classified into 40 classes using rotationally invariant K-mean clustering algorithm (33). Subaverages from nine major classes were used as references for multireference alignment (34). Cycles of refinement were performed 10 times. X-ray structures of the PSII core (3) and LHCII complex (6) (Protein Data Bank codes 3BZ1/3BZ2, and 2BHW, respectively) were manually fitted with PyMOL software (DeLano Scientific, San Carlos, CA) by reference to previous reports (16).

RESULTS

Isolation of the PSII-LHCII Supercomplexes from C. reinhardtii—In the present study, we solubilized the thylakoid membranes of wild-type C. reinhardtii with several different concentrations of either β- or α-DM (supplemental Figs. S3 and S4). When wild-type C. reinhardtii membranes solubilized with a low concentration of β-DM (0.8%) were subjected to SDG ultracentrifugation, three green bands appeared: A1 (representing free LHCII), A2 (representing the PSII core complex), and A3 (representing the PSI-LHCI supercomplex (Fig. 1). When the membranes were solubilized with a high concentration of β-DM (3.2%), an additional band (A2') appeared below the A3 band, and the A2 band disappeared (Fig. 1). When the membranes were solubilized with α-DM, the A2' band was more intense and shifted further below A3. The A2' band also appeared in a PSI-less mutant and a cytochrome b₅₆₇-f less mutant but not in a PSI-less mutant (Fig. 1), suggesting that it is related to the PSI complex and not the PSI or cytochrome b₅₆₇-f complex. We confirmed that by immunoblotting with anti-
bodies against PSI, PSII, and cytochrome \( b_{6}f \) complex subunits (Fig. 2). The intrinsic PSII subunits (D1 and CP47), the extrinsic PSII subunit (PsbO), the minor monomeric LHCIIIs (CP26 and CP29), and the major trimeric LHCII (LhcbM6) were detected predominantly in the A2' band (Fig. 2) whereas minor populations of PSII core subunits (D1 and CP47), PsbO, and LHClI subunits (CP26 and CP29) were detected in the A2 and A1 bands, respectively. PSI reaction center subunits (PsaA/B and PsAF) were detected only in A3, and a subunit for cytochrome \( b_{6}f \) complex (Cyt \( b_{6} \)) was detected only in A2. These results indicate that the A2' fraction contained both the PSI core complex and the LHClIIs, i.e. the PSII-LHClI supercomplex.

**Characterization of the PSII-LHClI Supercomplexes**—When we subjected the PSII-LHClI supercomplexes in the A2' fraction to gel filtration, those prepared with 3.2% B-DM had an estimated mass of 1.5 MDa whereas those prepared with \( \alpha \)-DM had an estimated mass of 1.0 MDa whereas those prepared with \( \beta \)-DM had an estimated mass of 1.5 MDa (Fig. 3A and Table 1). The 500-kDa difference corresponded to 3–4 additional LHClI trimers, assuming the mass of the LHClI trimers is \( \approx 150 \) kDa (16).

SDS-PAGE showed that the \( \beta \)-DM supercomplex, when normalized to the amount of D1 protein, bound less major (type I, III, and IV) and minor (CP26 and CP29) LHClIIs than the \( \alpha \)-DM supercomplex (Fig. 3B). Densitometric analysis determined that the \( \beta \)-DM supercomplex bound 1.5–2.7 times as less LHClII polypeptide as the \( \alpha \)-DM supercomplex (Table 2). Notably, the three luminal subunits of PSII (PsbO, PsbP, and PsbQ) were bound at a stoichiometric amount in the \( \alpha \)-DM supercomplex whereas most PsbP and PsbQ was lost in the \( \beta \)-DM supercomplex (Fig. 3B). Whereas the oxygen evolution rate of the \( \alpha \)-DM supercomplex was 378.8 \( \mu \)mol of \( \text{O}_2 \)/mg Chl per h in the presence of 10 mM CaCl\(_2\), which was 2.3 times that of the thylakoids (Table 3), the oxygen evolution rate of the \( \beta \)-DM supercomplex, which contained less LHClI polypeptides, was 174.6 \( \mu \)mol of \( \text{O}_2 \)/mg Chl per h, indicating that the \( \beta \)-DM supercomplex sacrificed oxygen evolution activity, likely because of the loss of PsbP and PsbQ.

The presence/absence of the luminal subunits and the integrity of the \( \text{O}_2 \)-evolving machinery in the two supercomplex preparations were further investigated by checking the effects of increasing Ca\(^{2+} \) concentration on the \( \text{O}_2 \) evolution activity. It has been reported that the spinach PSII particles lost all the three luminal subunits by washing with 1 mM CaCl\(_2\) resulting in total inactivation of \( \text{O}_2 \) evolution (35). Because the \( \text{O}_2 \)-evolving machinery was only slightly modified by CaCl\(_2\) wash, the \( \text{O}_2 \) evolution activity could be partially restored in a Ca\(^{2+} \) dependent manner (36). This is probably because the extra Ca\(^{2+} \) could substitute for the role of the luminal subunits (36) or protect the Ca atom within the M\(_n\)Ca complex (4). In supplemental Fig. S5, we showed that the oxygen evolution activity of the \( \beta \)-DM supercomplex was low in the presence of 10 mM CaCl\(_2\) (174.6 \( \mu \)mol of \( \text{O}_2 \)/mg Chl per h) but was elevated by increasing the CaCl\(_2\) concentration in a buffer up to 80 mM (353.4 \( \mu \)mol of \( \text{O}_2 \)/mg Chl per h), demonstrating that Ca\(^{2+} \) could restore the \( \text{O}_2 \) evolution activity of the \( \beta \)-DM supercomplex much as like that of the Ca-washed PSII particles (36), confirming that the functional binding of the luminal subunits was lost by the \( \beta \)-DM treatment. On the other hand, the apparently intact luminal subunits in the \( \alpha \)-DM supercomplex (Fig. 3B) were confirmed by the needlessness of the extra Ca\(^{2+} \) for its \( \text{O}_2 \) evolution activity (supplemental Fig. S5). These results demonstrate that the \( \alpha \)-DM supercomplex bound more LHClI polypeptides than the \( \beta \)-DM supercomplex and retained the integrity of the PSII reaction center including the luminal sub-
units, exhibiting a high oxygen evolution rate even under the low Ca\(^{2+}\)/H\(_{11001}\) conditions.

Because the part of the PSII core dimer (approximately 700 kDa) and the part of the LHCII antenna (approximately 800 kDa) could bind 70 (4) and 224 Chls, respectively, assuming that 42 Chls are bound to an LHCII trimer (150 kDa) (6), the entire \(\alpha\)-DM supercomplex could bind 294 Chls. Given that the \(\alpha\)-DM supercomplex, however, were wider and slightly longer (22 nm \(\times\) 35 nm, Fig. 4), because the length was close to that of \(\alpha\)-DM supercomplexes but the width was narrower than its dimer, these particles were more likely to be single supercomplexes with additional LHCII trimers than oligomers of the \(\alpha\)-DM supercomplexes.

Supplemental Fig. S2 shows top views projected from the luminal side of the eight classes of \(\alpha\)-DM supercomplexes (17 Å resolution). The central high density area corresponds to the luminal extrinsic subunits of the PSII core (shown in white in supplemental Fig. S2). The supercomplex with the largest area (414 particles) had three LHCII trimers (S, M, and L) on both sides of the core dimer, forming a \(\alpha\)-DM supercomplex bound 4.2 times as many Chls as the \(\alpha\)-DM supercomplex, its oxygen evolution rate (378.8 \(\text{mol of O}_2/\text{mg of Chl per h}\); Table 3) reflects 1.6 times higher specific activity per reaction center than that reported for the purified PSII core complex in \(\alpha\)-DM supercomplex obtained here retained its integrity. The oxygen evolution rate in the \(\beta\)-DM supercomplex, on the other hand, was somewhat lower (174.6 \(\mu\)mol of \(\text{O}_2/\text{mg of Chl per h}\); Table 3) even if it contains less Chl, indicating that the \(\beta\)-DM treatment for solubilizing PSII-LHCII supercomplex inactivated the \(\text{O}_2\) evolution activity.

**EM Structural Analysis of the PSII-LHCII Supercomplexes—**

The structures of the \(\beta\)-DM supercomplexes were consistent with those previously reported, where the particles were organized either as \(\alpha\)-DM supercomplexes, however, were wider and slightly longer (22 nm \(\times\) 35 nm, Fig. 4). Because the length was close to that of \(\alpha\)-DM supercomplexes but the width was narrower than its dimer, these particles were more likely to be single supercomplexes with additional LHCII trimers than oligomers of the \(\alpha\)-DM supercomplexes.

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plant PSII-LHClII supercomplexes with M- and/or L-trimers was that the position of CP24 in higher plants was replaced by L-trimers in *C. reinhardtii*. The previously proposed L-trimer positions in spinach were open in the *C. reinhardtii* supercomplex.

**DISCUSSION**

In earlier studies, Boekema *et al.* reported that the PSII-LHClII supercomplexes from spinach were organized as C2S2M1L1, C2S2M2L1, C2S2L1, or C2S2L2 (14). Their more recent study in *A. thaliana*, however, in which the C2S2M2 PSII-LHClII supercomplex was the one with the largest area, did not show the L-trimer associations (16). Even in spinach, the PSII-LHClII supercomplex with a stoichiometric amount of L-trimer, namely the C2S2M1L2 organization, which is presumably the most intact structure, was not observed, although its presence was hypothesized (14). In this study, PSII-LHClII supercomplexes with M- or L-trimers were observed for the first time in green algae, and the C2S2M1L2 supercomplex was observed for the first time in any organism. Notably, this PSII supercomplex associated with the largest number of LHClII trimers so far retained a high oxygen evolution activity. In *C. reinhardtii*, therefore, PSII could be associated with both M- and L-LHClII trimers despite the absence of CP24 (20).

As has been criticized by Nicholson *et al.* (39, 40) and more recently by Takahashi *et al.* (41) that detergent treatment may induce solubilization artifacts. This is an inherent problem in any studies by biochemical means, and we should continue to make efforts to check whether images obtained in vitro experiments correspond to those in vivo. Recently, strong lines of evidence have been reported to support that those detergent-solubilized single particles of the PSII-LHClII supercomplexes are indeed present in the thylakoid membranes. Daum *et al.* (18) identified the C2S2 structure in the osmotically shocked grana thylakoids in *A. thaliana* by cryoelectron tomography. Kouril *et al.* (17) performed cryoelectron tomography on the unsolubilized fraction of the digitonin-treated thylakoids from

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**TABLE 2**

Relative abundance of the polypeptides in the PSII-LHClII supercomplexes from α- and β-DM treated thylakoids

| Ratio between supercomplexes | D1 | CP26 | CP29 | Type I* | Type III* | Type IV* |
|-----------------------------|----|------|------|--------|----------|---------|
| PSII-LHClII (α-DM)/PSII-LHClII (β-DM) | 1.00 | 1.54 ± 0.03 | 2.70 ± 0.16 | 1.54 ± 0.05 | 1.37 ± 0.18 | 1.41 ± 0.08 |

* LHCII type I (LhcbM3, -4, -6, -8, -9).
* LHCII type III (LhcbM2, -7).
* LHCII type IV (LhcbM1).

**TABLE 3**

Characterization of the thylakoids, the PSII core complex, and the PSII-LHClII supercomplexes

| Preparation | Oxygen evolution rate per Chl | Estimated Chl content per PSII | Relative oxygen evolution rate per PSII* |
|-------------|-------------------------------|-------------------------------|-----------------------------------------|
| No CaCl2    | μmol O2 (mg Chl)⁻¹ h⁻¹       | μmol O2 (mg Chl)⁻¹ h⁻¹        | %                                      |
| Thylakoids  | 167.1 ± 1.2                   | ND                            | ND                                      |
| PSII core   | 1.030                         | 70                            | 100                                     |
| PSII-LHClII (α-DM) | 247.9 ± 18.1               | 294                           | 159                                     |
| PSII-LHClII (β-DM) | 103.8 ± 14.4              | 150                           | 37                                      |

* Calculation based on the estimated Chl content in the PSII core complex and LHClII proteins (see details in text).
* ND, not determined.
* Ref. 37.

**FIGURE 4.** Electron micrographs of PSII-LHClII supercomplexes in α-DM and β-DM. PSII-LHClII supercomplexes in α-DM (left) and β-DM (right) were loaded on glow-discharged carbon-coated copper grids and negatively stained with 2% uranyl acetate. Proteins are in white. The C2S2M1L2 particles are circled. Scale bar, 50 nm.
spinach to find the \( \text{C}_2\text{S}_2\text{M}_2\) particles. Therefore, it is more likely that the \( \text{C}_2\text{S}_2\text{M}_2\) structure identified in the current study exist in the thylakoids from \( \text{C. reinhardtii} \). Along the same line, we cannot rule out the possibility that more LHCII trimers are bound to PSII in \( \text{C. reinhardtii} \). Although particles larger than \( \text{C}_2\text{S}_2\text{M}_2\text{L}_2 \) supercomplexes have never been observed in any organisms, nor suggested by biochemical investigations, such giant complex may be found by using different solubilization procedures in future studies.

The most important difference between the \( \text{C}_2\text{S}_2\text{M}_2\text{L}_2 \) PSII-LHCII supercomplex described here and the one hypothesized for higher plants (14) is that the green algal L-trimers were found at the CP24 position in higher plants (Fig. 5). L-trimers, in contrast to S- and M-trimers, were bound directly to the PSII core (Fig. 5 and supplemental Fig. S2). Thus, energy harvested at the L-trimers could be transferred directly to the PSII core complex. Boekema et al. suggested that the strongly packed thylakoids (grana region) in higher plants, which contain crystalline PSII, have no space for L-trimer binding, but the non-PSII-crystalline (stroma-lamellae) region could have space for L-trimer binding (42). The weakly stacked thylakoids in \( \text{C. reinhardtii} \) (43, 44) might enable the L-trimer binding.

Because previous studies on the thylakoids in \( \text{C. reinhardtii} \) using \( \beta \)-DM reported only \( \text{C}_2\text{S}_2 \)-type organization (8, 19), it is likely that the binding of M- and L-trimers in \( \text{C. reinhardtii} \) is relatively weak, possibly due to the absence of CP24. That even S-trimer associations with the PSII core dimer are likely to be weaker in \( \text{C. reinhardtii} \) than in higher plants was suggested by the fact that S-trimer associations were observed only when membranes were treated with a high concentration of \( \beta \)-DM in both this study (3.2% \( \beta \)-DM) and in another (2.6% \( \beta \)-DM) (8). The absence of a PSII-LHCII supercomplex when we used low \( \beta \)-DM concentrations suggests that PSII-LHCII supercomplexes are less stable in \( \text{C. reinhardtii} \) than in higher plants. We speculate here that less stable supercomplexes are crucial for the migration of a large amount of mobile LHCIIIs during state transitions in green algae as it is in the CP24-lacking \( \text{Arabidopsis} \) (21). \( \text{C. reinhardtii} \) has a large capacity for state transitions (45), and almost 80% of its LHCIIIs can migrate between photosystems (46). It is thus possible that M- and L-trimers, as well as S-trimers, could dissociate easily from the PSII core, sustaining their high mobility during state transitions.

In conclusion, we have shown that \( \alpha \)-DM is more suitable than \( \beta \)-DM in terms of isolating large and active PSII-LHCII supercomplexes from green algae. The isolated PSII-LHCII supercomplex binds S-, M-, and L-LHCII trimers on each side of the reaction center. PSII-LHCII supercomplexes with M- or L-trimers were observed for the first time in green algae, and the \( \text{C}_2\text{S}_2\text{M}_2\text{L}_2 \) organization was observed for the first time in any organism. The methodology here should enable examination of the molecular details of PSII-antenna organization in several other photosynthetic eukaryotes, including diatoms, mosses, and plasinophytes. Moreover, purification of photosynthetic supercomplexes, including the PSII-LHCII supercomplex (this study), the PSI-LHCII supercomplex (24, 25), and the cyclic electron flow supercomplex (47) paved the way for challenging the studies on various photoacclimation events at the molecular level.

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Photosystem II Supercomplex in Chlamydomonas reinhardtii

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