Multimeric and monomeric photosystem II supercomplexes represent structural adaptations to low- and high-light conditions

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An intriguing molecular architecture called the “semi-crystalline photosystem II (PSII) array” has been observed in the thylakoid membranes in vascular plants. It is an array of PSII–light-harvesting complex II (LHCII) supercomplexes that only appears in low light, but its functional role has not been clarified. Here, we identified PSII–LHCII supercomplexes in their monomeric and multimeric forms in low light–acclimated spinach leaves and prepared them using sucrose-density gradient ultracentrifugation in the presence of amphipol A8-35. When the leaves were acclimated to high light, only the multimeric forms were present, suggesting that the multimeric forms represent a structural adaptation to low light and that disaggregation of the PSII–LHCII supercomplex represents an adaptation to high light. Single-particle EM revealed that the multimeric PSII–LHCII supercomplexes are composed of two (“megacomplex”) or three (“arraycomplex”) units of PSII–LHCII supercomplexes, which likely constitute a fraction of the semi-crystalline PSII array. Further characterization with fluorescence analysis revealed that multimeric forms have a higher light-harvesting capability but a lower thermal dissipation capability than the monomeric form. These findings suggest that the configurational conversion of PSII–LHCII supercomplexes may serve as a structural basis for acclimation of plants to environmental light.

Light is an important energy resource for photosynthetic organisms, but its quality and quantity are variable in the field (1). Photosynthetic organisms respond and acclimate to their light conditions by controlling their photosynthetic apparatus (2). In accordance with light intensities, light-harvesting, which is a primary process for photosynthesis, is optimized by modulating leaf and chloroplast movements and tuning the number of light-harvesting complex proteins in vascular plants (3, 4). Moreover, the organization of photosynthetic proteins in the thylakoid membranes is dynamically modulated according to light conditions. In vascular plants, a unique architecture called a semi-crystalline photosystem II (PSII) array is formed by PSII and light-harvesting complex II (LHCII) supercomplexes under low-light (LL) conditions, whereas the monomeric forms of PSII–LHCII supercomplexes are predominant under high-light (HL) conditions (5, 6). These configurational differences have been discussed as a potential strategy to overcome diffusion problems in the crowded conditions of thylakoid membranes (7) or to modulate the overall capacity for nonphotochemical quenching (NPQ) (8). However, because of technical limitations, the functional role of semi-crystalline PSII arrays has not been clarified.

NPQ, or qE quenching, is a photoprotection mechanism required to safely dissipate excessively absorbed light energy that otherwise tends to produce harmful reactive oxygen species (9, 10). For the last few decades, the physiological importance and molecular mechanisms of qE have been intensively studied. qE is triggered by lumenal acidification of the thylakoid membranes (9, 10). In vascular plants, compositions of the xanthophyll cycle pigments and PsbS are the crucial factors for qE (9). It has been proposed that a charge transfer within a chlorophyll (Chl) dimer and a zeaxanthin (Zea) or lutein (Lut) in minor LHCII proteins (11, 12) and excitation transfer from a Chl cluster to a Lut in LHCII proteins (13) contributes to qE quenching. There is an alternative model that does not involve carotenoids (Car), in which a charge transfer occurs within a Chl dimer in LHCII proteins (14). Acidification of the thylakoid lumen activates PsbS and violaxanthin de-epoxidase, converting violaxanthin (Vio) to Zea (9, 10). Both PsbS and Zea facilitate reorganization of PSII–LHCII supercomplexes and/or LHCII aggregation (10, 15, 16), suggesting that a configurational conversion of PSII–LHCII supercomplexes between a semi-crystalline array and a monomeric form might be linked to the modulation of NPQ (10).

Amphipols are amphipathic polymers that can be used as substitutes for detergents at the transmembrane surface of membrane proteins and thereby keep them soluble in detergent-free aqueous solutions (17). The most extensively studied and widely used APol, A8-35, is comprised of a mixture of short amphipathic polymers with a polycrylic backbone onto which two kinds of side chain have been randomly grafted: ~25% of its units carry an octylamide side chain, ~40% an iso-propylamide one, and ~35% an underv ativated carboxylate (18).

This article contains supporting information.
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Previously, we developed a procedure for purifying the PSII–LHCII supercomplex of Chlamydomonas reinhardtii employing A8–35 (19). Because the obtained complex, in which the detergent dodecyl-α-d-maltoside (α-DDM) used for its solubilization were replaced by A8–35, became much more stable (19), this A8–35-substituted preparation was successfully used to determine the structure by cryo-electron microscope (20) or the binding properties of the peripheral LHCII subunits (21).

Herein, we prepared PSII–LHCII supercomplexes from spinach leaves in their monomeric and multimeric forms in the presence of A8–35. Single-particle EM determined that the multimeric PSII–LHCII supercomplex, which was only identified in the LL-acclimated leaves, was composed of two or three units of PSII–LHCII supercomplexes like a fraction of the semi-crystalline array. It has a higher light-harvesting capability but a lower thermal dissipation capability than the monomeric form. We discuss possible implications of these results with respect to the functional role of the semi-crystalline array during environmental acclimation.

Results

Isolation of monomeric and multimeric PSII–LHCII supercomplexes using amphipol

We first isolated multimeric PSII–LHCII supercomplexes from LL-adapted spinach leaves. The thylakoid membranes were initially solubilized by α-DDM and applied to sucrose density gradient (SDG) ultracentrifugation in the presence of A8–35, which stabilized solubilized membrane protein complexes against thermodynamic dissociation (19), resulting in five green bands (Fig. 1A). Based on analysis by SDS-PAGE (Fig. 1B), the top three bands including A1, A2, and A3 were identified as free LHC proteins, PSI–LHCI supercomplexes/ATPases and PSII–LHCII supercomplexes, respectively. Although these three bands correspond to the bands previously observed in the study using a green alga C. reinhardtii, there were two additional bands present in the lower part (A4 and A5) (Fig. 1A). Because the polypeptide compositions in A4 and A5 were essentially identical to that of A3 (Fig. 1B), we presumed that the A4 and A5 bands corresponded to multimers of PSII–LHCII supercomplexes. Interestingly, the lower two bands (A4 and A5 bands) were not detected in the sample prepared from the HL-treated leaves, whereas the upper three bands (A1, A2 and A3) stayed the same (Fig. 1A), suggesting that the putative multimeric PSII–LHCII supercomplexes (A4 and A5) were only present in the leaves acclimated to LL conditions. The LL-dependent formation of the possible multimeric forms of PSII–LHCII supercomplexes was reminiscent of the formation of semi-crystalline PSII arrays observed in LL-adapted leaves (6).

The structure of monomeric and multimeric PSII–LHCII supercomplexes

PSII configurations in A3, A4, and A5 bands were characterized by single-particle analysis of EM of negatively stained particles (Fig. 1C and Fig. S1). As expected from the polypeptide compositions, the A3 band was composed of monomers of PSII–LHCII supercomplexes, whereas the A4 and A5 bands were composed of multimers of PSII–LHCII supercomplexes.

Four classes of monomeric PSII–LHCII supercomplexes were observed in the A3 band (Fig. S2). Ten classes of PSII–LHCII supercomplex pairs were observed in the A4 band (Fig. S3), which has been reported as “PSII–LHCII megacomplexes” in the literature (22). Twelve classes of an array of three PSII–LHCII supercomplex units were observed in the A5 band (Fig. S4), which has never been reported before (hereafter referred to as “PSII–LHCII arraycomplexes”). In other literature, similar bands to the A4 and A5 bands have been isolated by using α-DDM, and these bands were identified as (C2S2)4 and (C2S2M2)2 formed by “sandwiched” PSII–LHCII supercomplexes (23, 24). However, these sandwiched complexes were absent in the isolation using amphipol (20, 25). In the A3 band, a C2S2M1-type PSII–LHCII supercomplex composed of a core dimer complexes (C3) with two strongly bound LHCII trimers (S2) and one moderately bound LHCII trimer (M1) was dominant (50%), which was consistent with the previous report in spinach (26). The configuration of the PSII–LHCII megacomplexes in the A4 band here is similar to the previously reported PSII–LHCII megacomplexes from Arabidopsis thaliana (23). In the A5 band, PSII–LHCII arraycomplexes form various types of alignments much as like the sections in a semi-crystalline array (26) (Fig. S5), suggesting the possibility that the A4 and A5 bands were fractions of a larger, semi-crystalline PSII array.

Pigment compositions

To characterize optical properties and pigment composition, we obtained the absorption spectrum of each band (Fig. S6A). Two main peaks at ~435 and ~675 nm originated from Chl a, the shoulder ~650 nm originated from Chl b and the shoulder at ~475 nm originated from both Chl b and Car. Thus, higher absorption at 475 nm but similar absorption level at 650 nm in the A4 and A5 bands than that in the A3 band represents the larger contributions of Car. This result is consistent with the pigment analysis using ultra-performance liquid chromatography (Table S1), showing that A4 and A5 have higher Car content (Lut, Vio, and neoxanthin) per Chl a than the A3 band. Another feature of A4 and A5 is that it has a red-shifted peak at ~676 nm compared with A3. This is most likely due to contamination by PSI because PSI shows a red-shifted absorption peak at 679.5 nm. This was supported by immunoblotting analysis of the amount of PsaA, in which the A4 and A5 bands contained 29 and 20% more PSI per PSII (D1) than the A3 band (Fig. S7). To further characterize the PSII–LHCII supercomplexes, we thus adopt Chl fluorescence measurements at room temperature because most of fluorescence comes from PSII at room temperature (Fig. S6B) (27).

Quenching properties

To assess the photoprotection capabilities, we analyzed quenching properties of A3, A4, and A5 by comparing Chl fluorescence decay at neutral (pH 7.5) and acidic pH (pH 5.5). NPQcalc (28) was derived from fluorescence lifetimes of the non-quenching form (at pH 7.5) and the quenching form (at pH 5.5) and was used as a Stern–Volmer type NPQ parameter. As shown in Fig. 2A, the fluorescence lifetime of A3 was shorter...
than A4 and A5 when they were at acidic pH. NPQcalc of A3 was 0.79, whereas those of A4 and A5 were 0.22 and 0.27, respectively (Table 1), suggesting that the monomeric PSII–LHCII supercomplexes had a much higher qE capability than the multimeric PSII–LHCII supercomplexes when in the low pH. To identify the origin of the higher qE capability in the monomeric PSII–LHCII supercomplexes, the amount of PsbS was compared because it has been reported that the PsbS could contribute to qE by associating with PSII–LHCII supercomplexes (29, 30). The amount of PsbS per PSII core complex (D1) was comparable in all three samples (Fig. S7), indicating that the elevated quenching capability in A3 was independent of PsbS. On the contrary, more Car was bound per Chl a to A4 and A5 than A3 (Table 1). A4 and A5 possessed 5 and 16% more Lut, and 13 and 24% more Vio per Chl a than A3, respectively. Because we analyzed the leaves adapted to LL conditions, Zea was not detected in all samples, suggesting that the elevated quenching capability of A3 here was independent of Zea. These results indicate that the higher qE capability in A3 was not due to the amount of PsbS nor Car but could be due to conformational changes caused by monomerization of PSII–LHCII supercomplexes such as changes in the orientations of Chl(s) or Car(s). It has actually been reported that conformational changes by protein–protein interactions can activate qE process (13, 31, 32).

**Light-harvesting capabilities**

The light-harvesting capabilities of monomeric and multimeric PSII–LHCII supercomplexes were investigated by fluorescence induction traces upon dark-to-light transition according to the previous method (33) (Fig. 3A), which determined the cross-section for PSII excitation (PSII cross-section). Because a heterogenous puddle model yielded a better fit than a

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**Figure 1. Isolation and structural characterization of photosystem complexes.** A, SDG ultracentrifugation of the solubilized thylakoid membranes isolated from LL-adapted leaves and after HL treatment (1000 μE m⁻² s⁻¹) for 1, 2, 4, 8, and 16 h. Thylakoid membranes isolated from spinach were solubilized with α-DDM and then replaced by amphipol A8-35 during SDG ultracentrifugation. B, polypeptides in the SDG fractions of LL-adapted leaves in A analyzed by SDS-PAGE stained by Coomassie Brilliant Blue R-250. C, averaged 2D projection maps of negatively stained particles in A3, A4, and A5 band from the SDG fractions of LL-adapted leaves in A. 2266, 2296, and 2626 particles, respectively, for A3, A4, and A5 bands were obtained and analyzed by three independent biological repetitions. The four most abundant classes are shown, and remaining classes are shown in Fig. S2–S4. The percentages in 2D projection maps represent the relative abundance of the particles in each band. Scale bar, 10 nm.
The error ranges represent standard error (n = 4, biological replicates).

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>τ₁</th>
<th>d₁</th>
<th>τ₂</th>
<th>d₂</th>
<th>τ₃</th>
<th>d₃</th>
<th>τ_{ave.int}</th>
<th>NPQ_{calc}^ab</th>
</tr>
</thead>
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<tr>
<td>A3</td>
<td>7.5</td>
<td>0.20</td>
<td>0.80</td>
<td>0.82</td>
<td>0.18</td>
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<td>0.02</td>
<td>0.87 (±0.07)</td>
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<tr>
<td></td>
<td>5.5</td>
<td>0.15</td>
<td>0.86</td>
<td>0.59</td>
<td>0.13</td>
<td>2.15</td>
<td>0.01</td>
<td>0.49 (±0.02)</td>
</tr>
<tr>
<td>A4</td>
<td>7.5</td>
<td>0.23</td>
<td>0.71</td>
<td>0.90</td>
<td>0.26</td>
<td>2.57</td>
<td>0.03</td>
<td>0.95 (±0.02)</td>
</tr>
<tr>
<td></td>
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<td>0.77</td>
<td>0.79</td>
<td>0.21</td>
<td>2.41</td>
<td>0.02</td>
<td>0.77 (±0.02)</td>
</tr>
<tr>
<td>A5</td>
<td>7.5</td>
<td>0.27</td>
<td>0.64</td>
<td>0.98</td>
<td>0.33</td>
<td>2.56</td>
<td>0.02</td>
<td>0.92 (±0.01)</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>0.23</td>
<td>0.74</td>
<td>0.84</td>
<td>0.24</td>
<td>2.34</td>
<td>0.02</td>
<td>0.73 (±0.02)</td>
</tr>
</tbody>
</table>

^aτ_{ave.int} represent intensity-weighted average.

^bNPQ_{calc} was derived as \( \tau_{ave.int at pH 7.5} / \tau_{ave.int at pH 5.5} \).
to the environmental light conditions, which eventually contributes to photosynthesis yield (34).

The other important issue arisen from this study is the factor triggering the configurational modulation of PSII–LHCII supercomplexes. We hypothesize four factors: (i) acidic pH, (ii) phosphorylation of LHC and/or PSII core, (iii) the activation of PsbS, and (iv) accumulation of Zea. (i) Our previous study (21) showed that the acidic pH condition induces a conformational change of the complexes so that binding of the S-trimer to the PSII core becomes loose. We suppose that such HL-adapted conditions induce sufficiently long acidic conditions, which can induce the conformational change. (ii) It has been well-studied that the STN7- and STN8-dependent phosphorylation induces detachment of LHCII trimers from PSII and reorganization of photosynthetic protein complexes (35, 36). We suppose that such a large reorganization can induce configurational modulation of PSII–LHCII supercomplexes. (iii) It has been shown that PsbS regulates the membrane fluidity, which is an important factor for reorganization of photosynthetic protein complexes (15). Therefore, it is plausible that the activation of PsbS induces a configurational modulation of PSII–LHCII supercomplexes (8). (iv) It is well-known that the HL condition induces de-epoxidation of Vio (37, 38). We suppose that a change of xanthophyll species can induce the configurational modulation of PSII–LHCII supercomplexes because of its different chemical structure and property. These hypotheses can be verified by further study using several mutants lacking PsbS, STN7, or STN8 and the xanthophyll cycle.

Our findings provide a feasible explanation for the discovery of Goral et al. (39) that showed a correlation between semi-crystalline PSII arrays in vivo and reduced NPQ in LHCII and PsbS mutants in Arabidopsis. This could be at least partially explained by the reduced mobility of the PSII components and restricted freedom for the rearrangement that is required for a transition into the NPQ state, when in the crystalline arrangement. The details of the molecular mechanism of how this configurational change occurs remains to be investigated by structural and ultrafast spectroscopic studies. Determination of the atomic structures of the multimeric complexes described here would also be important to discuss by comparing the LHCII heterogeneity reported recently (24).

### Experimental procedures

**Isolation of thylakoid membranes and photosystem complexes**

Spinach leaves were quickly frozen in liquid nitrogen after light treatments and disrupted using a blender with a buffer containing 0.33 M sucrose, 5 mM MgCl2, 1.5 mM NaCl, and 25 mM MES (pH 6.5, NaOH). From the suspension obtained by blending, thylakoid membranes were isolated according to the same protocol used for C. reinhardtii (40) and were suspended in a 25 mM MES buffer (pH 6.5) containing 1 M betaine at 0.3 mg Chl/ml and solubilized with 1.0% α-DDM (Anatrace, Maumee, OH, USA) for 10 min on ice. Unsolubilized thylakoids were removed by centrifugation at 25,000 g for 1 min. After centrifugation, A8-35 (Anatrace) was added at a final concentration of 1% to the solubilized thylakoids for amphipol substitution and subjected to SDG ultracentrifugation as previously described (19). For spectroscopic analysis of isolated PSII–LHCII complexes, the samples were diluted to 2 μg/ml Chl by a 25 mM MES buffer (pH 6.5) containing 1 M betaine.

**SDS-PAGE and immunoblotting analysis**

SDS-PAGE and immunoblot analyses were performed as previously described (41). For Coomassie Brilliant Blue R-250 staining, 1.0-μg Chl samples were applied to SDS-PAGE. For immunoblot analyses, 0.3-μg Chl samples were applied to SDS-PAGE. Antibodies for D1 and PsbS proteins were purchased from AgriSera (AS05-084 for D1 and AS09-533 for PsbS). The antibody used for PsA detection was described previously.
An anti-rabbit horseradish peroxidase–conjugated antiserum (#7074, Cell Signaling Technology, Danvers, MA, USA) was used as a secondary antibody. Densitometric analyses of the detected images were performed using Image laboratory software (Bio-Rad).

**EM and single-particle analysis**

The isolated complexes were diluted to 2 μg/ml Chl in the 25 mM MES buffer, applied to glow-discharged carbon-coated copper grids, and negatively stained for 30 s with 2% uranyl acetate three times. Electron micrographs were recorded using an Olympus Velete CCD camera (2,048 × 2,048) at a pixel size of 5.0 Å. In total, 100 micrographs for PSII–LHCII supercomplexes, 400 micrographs for PSII–LHCII megacomplexes, and 600 micrographs for PSII–LHCII arraycomplexes were collected. The Relion 2.1 package was used for 2D classification (42).

**Pigment analysis**
Pigments of PSII supercomplexes extracted by 80% acetone and analyzed by ultra-performance liquid chromatography using the H-class system (Waters) as previously described (43).

**Time-resolved fluorescence decay analysis**

Time-resolved fluorescence decay traces were obtained by a time-correlated single photon counting system (FluoRcube; HORIBA Jobin-Yvon) at room temperature. Samples were excited at 441 nm with a repetition rate of 1 MHz by a diode laser (N-440L; HORIBA Jobin-Yvon), and emissions were detected at 685 nm with 32-nm bandwidths.

**Fluorescence induction traces measurements and analysis**

Prior to the measurement of fast fluorescence induction traces, PSII–LHCII complexes were kept in the dark for 6 h for fully oxidizing PSII reaction centers \( (F_0) \). Fast fluorescence induction traces, from \( F_0 \) (open reaction centers) to \( F_m \) (closed reaction centers), were measured under weak light conditions (28 μE m\(^{-2}\) s\(^{-1}\)) in the presence of 20 μM 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU). The sample concentrations were set to 10 μg/ml Chl. The concentration of DCMU has been set as enough concentration for saturated conditions (33).

To simulate the fast fluorescence induction of the samples, we used the procedure previously outlined in the supplementary information of a previous study (33), which was based on a relevant mathematical model (44). Here, as in the previous study, it was found that a satisfactory fit is only obtained for a heterogeneous puddle model. Indeed the lake model can be discounted by simple visual inspection of the traces because of their obvious lack of sigmoidicity. In the puddle model each antenna puddle contains a finite (and small) number of reaction centers (RCs), \( N \). A full derivation of the overall theory can be found in the previous study (33). Here, we briefly summarize the key equations. The normalized fluorescence induction trace, in the presence of qE quenchers, is defined as follows,

\[
\Phi(t) = \frac{1 + Q - p}{p} \left[ 1 + Q \sum_{j=0}^{N} \frac{P_j(t)}{1 + Q - p \frac{N}{j}} - 1 \right] \quad \text{(Eq. 1)}
\]

where

\[
Q = \frac{k_{qE}}{k_0} \quad \text{(Eq. 2)}
\]

is the ratio between the rate constants of excitation trapping by the qE quenchers, \( k_{qE} \), and excitation trapping by open RCs, \( k_0 \). Similarly,

\[
p = \frac{k_c}{k_0} = 1 - \frac{F_0}{F_m} \quad \text{(Eq. 3)}
\]
describes (background) quenching in conditions of closed reaction centers. The summation index \( i \) counts the number of closed RCs. For each value of \( i \), we assign a time-dependent probability, \( P_i(t) \). These probabilities evolve according to the following master equations,

\[
\frac{dP_0}{dt} = -k_0n_0P_0 \quad \text{(Eq. 4)}
\]

\[
\frac{dP_i}{dt} = k_0 \left[ (1 - \frac{i-1}{N})n_{i-1}P_{i-1} - \left( \frac{i}{N} \right)n_iP_i \right], \quad 1 \leq i \leq N-1 \quad \text{(Eq. 5)}
\]

\[
\frac{dP_N}{dt} = k_0 \left( \frac{N-1}{N} \right)n_{N-1}P_{N-1} \quad \text{(Eq. 6)}
\]

where \( n_i \) is the steady-state excitation density within a system with \( i \) closed RCs,

\[
n_i = \frac{G}{k_0} \left( \frac{1}{1 + Q - p \frac{N}{N}} \right) \quad \text{(Eq. 7)}
\]

and \( G \) is the rate constant for excitation generation in the antenna assuming constant light intensity, \( I \). It is directly proportional to the cross-section, \( \sigma \), of the antenna,

\[
G = \sigma I \quad \text{(Eq. 8)}
\]

As \( N \) increases, lake-type (sigmoidal) behavior is recovered. The best fits were obtained for \( N = 2 \) (i.e. a PSII RC dimer) in all cases. Moreover, the traces are visibly biphasic, and so good fits could only be obtained if we assumed heterogeneity. For simplicity we assumed two subpopulations. The fluorescence trace is then given by,

\[
\Phi(t) = \sum_{j=1}^{2} h_j \Phi_j(t) \quad \text{(Eq. 9)}
\]

where

\[
h_1 + h_2 = 1 \quad \text{(Eq. 10)}
\]

In total, our fitting parameters are \( G_1, G_2, h_1, Q_1 \), and \( Q_2 \). The parameter \( p \) is obtained directly from the data and is assumed to be identical for each subpopulation and independent of pH. To avoid overfitting, we adopted a careful procedure. First we fit the traces for pH 7.5 and assume that \( Q_1 = Q_2 = 0 \). Any residual quenching present is quantified by \( p \). Next, the long-time kinetics (i.e. as the curve approaches the maximal fluorescence yield) are defined almost entirely by the subpopulation with the smallest cross-section, which we define as \( G_1 \). We therefore obtain \( G_1 \) by fitting the long-time data to,

\[
\Phi(t \to \infty) \approx 1 - e^{-bt} \quad \text{(Eq. 11)}
\]

where

\[
b = \frac{G_1}{N - p(N - 1)} \quad \text{(Eq. 12)}
\]


