Novel Features of Eukaryotic Photosystem II Revealed by Its Crystal Structure Analysis from a Red Alga\*\*\*


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Photosystem II (PSII) catalyzes light-induced water splitting, leading to the evolution of molecular oxygen indispensable for life on the earth. The crystal structure of PSII from cyanobacteria has been solved at an atomic level, but the structure of eukaryotic PSII has not been analyzed. Because eukaryotic PSII possesses additional subunits not found in cyanobacterial PSII, it is important to solve the structure of eukaryotic PSII to elucidate their detailed functions, as well as evolutionary relationships. Here we report the structure of PSII from a red alga Cyanidium caldarium at 2.76 Å resolution, which revealed the structure and interaction sites of PsbQ’, a unique, fourth extrinsic protein required for stabilizing the oxygen-evolving complex in the lumenal surface of PSII. The PsbQ’ subunit was found to be located underneath CP43 in the vicinity of PsbV, and its structure is characterized by a bundle of four up-down helices arranged in a similar way to those of cyanobacterial and higher plant PsbQ, although helices I and II of PsbQ’ were kinked relative to its higher plant counterpart because of its interactions with CP43. Furthermore, two novel transmembrane helices were found in the red algal PSII that are not present in cyanobacterial PSII; one of these helices may correspond to PsbW found only in eukaryotic PSII. The present results represent the first crystal structure of PSII from eukaryotic oxygenic organisms, which were discussed in comparison with the structure of cyanobacterial PSII.

Oxygenic photosynthesis provides us with food, oxygen, and fuel and is therefore vital to life on the earth. The first reaction occurring in oxygenic photosynthesis is the splitting of water into electrons, protons, and molecular oxygen, among which electrons and protons are utilized for the synthesis of NADPH and ATP, whereas oxygen is supplied to the atmosphere for maintaining aerobic life forms. The water splitting reaction is catalyzed by photosystem II (PSII), an extremely large membrane-protein complex located in thylakoid membranes from prokaryotic cyanobacteria to higher plants. In the case of cyanobacteria, the crystal structure of PSII has been solved with its resolution gradually increased to an atomic level of 1.9 Å (1–6), which showed that PSII contains 17 transmembrane subunits and 3 peripheral, hydrophilic subunits with a total molecular mass of 700 kDa for a dimer.

The first oxygenic photosynthetic organism is believed to be the ancestor of cyanobacteria some 2.7 billion years ago (7). Although the subunit compositions of PSII from cyanobacteria to higher plants we see today are rather conserved, some apparent differences exist in both the transmembrane and peripheral subunits among cyanobacteria, various algae, and higher plants (8). One of the remarkable differences is found in the composition and function of extrinsic proteins associated in the lumenal side and required for maintaining the optimal function of the water-splitting reaction. In cyanobacteria, three extrinsic proteins of PsbO (33 kDa), PsbU (12 kDa), and PsbV (17 kDa, cytochrome c550) are associated with purified PSII (9–11), and their structures and locations have been revealed in the crystal structure of PSII (2–6, 12). Among these three proteins, the PsbO protein is found in PSII of all oxygenic organisms ranging from...
cyanobacteria to higher plants. The other two subunits, PsbU and PsbV, are present in PSII of cyanobacteria and most eukaryotic algae including red algae, diatom etc (11, 13–15), whereas they are replaced by two nonhomologous subunits, PsbQ (17 kDa) and PsbP (23 kDa), in the green lineage including green algae and higher plants (11, 15–20). Proteins homologous to PsbQ and PsbP were also found in cyanobacteria (cyano-PsbQ and cyano-PsbP) (15–21), as well as some eukaryotic algae such as diatom (14). The cyanoQ and cyanoP, however, have been lost in the purified cyanobacterial PSII, and no crystal structure of PSII from eukaryotic organisms has been solved; therefore, the association pattern of these proteins with PSII is not clear. In addition to these differences, there are also some differences in the subunit composition of small transmembrane proteins. For example, the PsbW subunit is found in green algal and higher plant PSII but not in cyanobacterial PSII, whereas its presence in lower eukaryotic algae is not clear.

Red algal PSII is unique in that it contains four extrinsic subunits, three of which are cyanobacteria type, whereas the fourth one is PsbQ′, an extrinsic protein with a low homology to PsbQ found in the green lineage (11, 13, 15, 16, 22). The function of this protein was found to be facilitating the binding of PsbU and PsbV to PSII, as well as regulating the requirements of oxygen evolution for Cl− and Ca2+, two indispensable cofactors for oxygen evolution (11, 13). Because of the presence of this protein, the binding mode of PsbV was found to be different between cyanobacterial and red algal PSII: although PsbV is able to bind to cyanobacterial PSII independent of other extrinsic proteins, it binds to red algal PSII effectively only in the presence of PsbQ′ (10, 13). The binding position and structure of PsbQ′ in red algal PSII, however, is not clear at present.

To elucidate the structural differences between PSII from cyanobacteria and eukaryotic photosynthetic organisms, it is essential to analyze the structure of eukaryotic PSII. No structures of PSII from eukaryotic organisms, however, have been solved so far, partly because of the instability of eukaryotic PSII upon isolation, which is largely resulted from the loose association of the extrinsic proteins in eukaryotic PSII. Because crystal contact within the cyanobacterial PSII crystals are largely mediated by hydrophilic surfaces contributed by extrinsic subunits, loss of some of these subunits will result in the failure of formation of the crystal contact. Thus, it is important to use PSII particles with all of its extrinsic subunits associated. Previously, we reported the crystallization of PSII from an acido-phobic, thermophilic red alga Cyaniidium caldarium (23), the first eukaryotic alga with its PSII successfully crystallized.

**Crystal Structure of Eukaryotic Photosystem II**

**Experimental Procedures**

**Purification and Crystallization of the Red Algal PSII**—Cells of *Cyaniidium caldarium* were grown at 42 °C for 12–15 days under bubbling of air containing 3–5% CO2. The harvested cells were broken by glass beads, and oxygen-evolving PSII core complexes were purified from the thylakoid membranes according to the procedures described previously (13, 23, 24) with slight modifications where n-dodecyl-α-D-maltoside (α-DDM) was used to solubilize the thylakoid membranes instead of n-dodecyl-β-D-maltoside (β-DDM) used previously. All of the solutions in the subsequent column purification and resuspension of the PSII cores included α-DDM in place of β-DDM.

Crystallization of the purified PSII cores was performed with the conditions described previously (23), with α-DDM replacing β-DDM as the detergent, and PEG 1450 as the precipitant. For improving the purity and homogeneity of the sample, a recrystallization step was introduced in which microcrystals were grown overnight at 0 °C. The microcrystals obtained were collected by centrifugation, redissolved, and utilized for the second crystallization with the oil batch method. Large crystals were obtained in 7–10 days at 4 °C.

Crystals grown to a size of 0.6 mm × 0.3 mm × 0.1 mm were transferred to a cryoprotectant solution containing 10% glycerol and 35% PEG 3000 in the crystallization buffer by dialysis for 3 days at 4 °C using a 50-µl dialysis button (Hampton Research). To improve the crystal resolution, the crystals were dehydrated in 50 µl of cryoprotectant solution against a saturated NaBr humidity for 4 h at 20 °C after dialysis, and then flash frozen in a nitrogen gas stream at 100 K. To obtain heavy atom derivatives, crystals were soaked in heavy atom solutions containing 45% PEG 3000, 10% glycerol, and 1–2 mM heavy atoms for 6–24 h after dehydration.

**Data Analysis and Structure Determination**—X-ray diffraction data were collected at Beamlines BL41XU and BL44XU of SPring-8 (Japan) and processed by the software XDS. The initial phase was calculated with SOLVE (25) at 4.5 Å resolution by the multiwavelength anomalous diffraction phasing method with the diffraction data obtained from a Ta6Br14 derivative crystal, TaBr-1: peak (λ = 1.2548 Å); TaBr-2: inflection (λ = 1.2552 Å); TaBr-3: high remote (λ = 1.2303 Å) (see Tables 1–3). The binding sites of tungsten, mercury, and the TaBr12 cluster were determined by the difference Fourier synthesis between the native and each of the derivatives with the initial phase. The refinement of all heavy atom parameters and the calculation of multiple isomorphous replacement-anomalous signal combined phase at 3.5 Å were performed using SHARP (26), and then the phase information was extended to 2.9 Å resolution by RESOLVE (27). Initial structural model was built with COOT (28) manually using protein sequences of *C. caldarium* where it is available and the sequences of a closely related red alga Cya-nidioschyzon merolae when that from *C. caldarium* was not available. The structure was finally refined to 2.76 Å resolution using PHENIX (29). The two transmembrane helices not found in cyanobacterial PSII were designated with chain ID S and W and were basically modeled as polyalanines. A few amino acid residues having large side chains visible in the electron density map of the two helices were modeled as amino acid residues.
Mass Spectrometric Analysis—For identification of the protein subunits in the red algal PSII, we purified PSII from C. merolae with the same procedures used for cultivation and purification of PSII from C. caldarium, because only the genome sequence of C. merolae is available. The PSII complexes were participated by a solution of acetone/ethanol/acetic acid (50:50: 0.1) and stored at −30 °C until usage. For bottom-up analysis, the protein was redissolved into 8 M urea and 50 mM Tris-HCl buffer (pH 8) followed by diluting to 1 M urea with 50 mM Tris-HCl buffer (pH 8), and then digested with either trypsin or chemotrypsin. For top-down analysis, the protein was redissolved into 10% formic acid aqueous solution. Both peptides and intact proteins were subjected to LC-MS/MS analysis using a Thermo Ultimate 3000 nano LC system coupled with a Thermo LTQ-Orbitrap Elite.

The digested peptides were separated with a homemade 20-cm × 75-μm C18 capillary column assisted with a 5-cm × 200-μm C18 trap column. The peptides were eluted by a gradient of 5–40% buffer B (acetonitrile containing 0.1% formic acid) in 120 min. The mass spectrometry data were collected with a data-dependent mode. The MS1 spectra were collected in a range of 380–1800 m/z with a resolution of 120 K. The top 20 ions in MS1 were subjected to high-energy collisional dissociation fragmentation with a normalized collision energy of 25, and the corresponding fragment ions were collected with a resolution of 30 K. The raw data were processed with Thermo Protein Discoverer (version 1.4.0.288) with the Sequest HT search engine. The C. merola database from Uniprot was applied with the oxidation of methionine as the only dynamic modification. The corresponding results were filtered with a false discovery rate of 0.01.

For intact proteins, a homemade 20-cm × 75-μm C5 capillary column and a 5-cm × 200-μm C5 trap column were utilized. The column was eluted by a gradient of 20–100% buffer B in 90 min and followed by an isocratic elution at 100% buffer B for 10 min. The MS1 and MS/MS spectra were collected by Orbitrap with resolutions of 120 and 60 K, respectively. The number of micro scans for both MS1 and MS/MS was set to 5. The top five ions in MS1 were fragmented by high-energy collisional dissociation with a normalized collision energy of 35. To shorten the search time, protein identification were performed by searching each raw files against a simplified PSII protein database of C. merolae (Uniprot) using ProSight PC 3.0 (Thermo Scientific). The precursor and fragment ions were first deconvoluted with an embedded Thrash algorithm. The precursor with a mass above 750 and more than 10 fragments was subjected to search. The fragment mass tolerance was set as 15 ppm. The false discovery rate (below 0.01) was controlled with an E value below 1E-4.

Results

Structure Determination and the Overall Structure of the Red Algal PSII—Previously we have obtained two types of PSII crystals from the red alga C. caldarium; one has a space group of P2221 with a resolution of 3.8 Å, and the other one has a space group of P2221, with a resolution of 3.5 Å (23). To improve the crystal resolution, we used α-DDM to solubilize, purify, and crystallize the PSII sample instead of β-DDM. The protein composition of the PSII samples we obtained is shown in Fig. 1. In comparison with the previous samples obtained with β-DDM (23), no apparent differences were found in the protein composition either before or after crystallization. This is confirmed by our MS analysis (see below and supplemental Table S1), which showed that all of the PSII components were found in our purified PSII. We also confirmed that the α-DDM-purified PSII had an oxygen evolving activity of 3000–4000 μmol O2/mg Chl/h, which is compatible with that of β-DDM-purified PSII.

By optimizing the crystallization and postcrystallization dehydration conditions, we were able to improve the resolution of the second type crystal to a resolution of 2.76 Å (Tables 1–3). It was found that the red algal PSII exists in a tetramer within the crystal, which is different from the dimeric cyanobacterial PSII found in both solution and crystals (1–6, 30). Attempts to solve the crystal structure by the molecular replacement method with the cyanobacterial PSII as the search molecule was not successful, probably because of (i) the tetrameric packing of red algal PSII versus dimeric packing of cyanobacterial PSII; (ii) the extremely large molecule mass of 1,400 kDa for a tetrameric PSII; and (iii) limited resolution of the diffraction data. Thus, we collected diffraction data from several heavy atom derivatives to obtain the phase information and used the multiple isomorphous replacement-anomalous signal method to solve the structure (Tables 1–3). The structure obtained showed Rwork/Rfree values of 0.245/0.274 and an overall temperature factor (B-factor) of 69.2 Å2 for protein atoms (Tables 1–3). A few water molecules were assigned in addition to the protein chains and large number of
cofactors. A part of the electron density map was shown in Fig. 2, which demonstrated that the quality of the map obtained is good enough to enable us to assign most of the protein chains.

The overall structure of a PSII tetramer is shown in Fig. 3A, from which it is clear that two dimers are stacked through their stromal surfaces to form the tetramer. This is probably due to the fact that the stromal side is rather "flat," making the contact between them possible. The two dimers are rotated (twisted) ~20° with respect to each other. Despite this twist, most of the surface area in the stromal side between the two dimers were stacked, giving rise to enough surface area for the interaction of the two dimers to form the tetramer. Because the red algal PSII existed in a dimeric form in solution (before crystallization) (23), it can be concluded that the tetramization occurred within the crystal, probably because of the requirements imposed by the crystal packing. Because the space group of the crystal is $P2_12_12_1$, there are four tetramers in a unit cell within the crystal.

The overall structure of each dimer is similar to that of the cyanobacterial PSII (Fig. 3, B and C), except the presence of additional subunits (see below). The root mean square deviation values were calculated to be 0.731–0.796 Å when the Ca atoms of each monomer of the red algal PSII tetramers were superimposed with a cyanobacterial PSII monomer. In total, 22 protein subunits were found in each monomer, among which, 18 are transmembrane subunits, and 4 are peripheral, hydrophilic subunits. The transmembrane subunits include 16 proteins that are found in the cyanobacterial PSII (PsbA, PsbB, PsbC, PsbD, PsbE, PsbF, PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbT, PsbX, PsbZ, and Ycf12 (Psb30)) (Table 4). Among these subunits, the PsbZ subunit was present in two monomers (the four monomers in the tetramer were designated Mol-1, Mol-2, Mol-3, and Mol-4 (Fig. 3A) and PsbZ was present in Mol-3 and Mol-4) but absent in the other two monomers (Fig. 4 and Table 5). The loss of PsbZ in Mol-1 and Mol-2 in the crystal is most likely due to the effect of crystal packing and also suggests that the association of PsbZ with other subunits of PSII is rather weak, resulting in the easy loss of this subunit. The PsbY subunit was also not present in the crystal structure, which is similar to the case of cyanobacterial PSII, with the interatomic distances restrained from the cyanobacterial high resolution structure. A detailed comparison between the two structures of OEC from the red alga and cyanobacterium is not possible because of the limited resolution of the red algal PSII structure. Numbers of two to seven

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**TABLE 1**

<table>
<thead>
<tr>
<th>Data collection and phasing for the red algal PSII crystals</th>
<th>Tungsten</th>
<th>Mercury</th>
<th>Tantalum-1</th>
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<td>Data set</td>
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<td>Ta$<em>{Br</em>{4}}$, remote</td>
<td>Ta$<em>{Br</em>{4}}$, peak</td>
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<td>Space group</td>
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<td>$P2_12_1$</td>
<td>$P2_12_1$</td>
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<td>Cell dimensions: $a$, $b$, $c$ (Å)</td>
<td>210.4, 240.3, 300.1</td>
<td>209.2, 235.9, 299.8</td>
<td>209.7, 238.3, 299.5</td>
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<tr>
<td>Resolution (Å)</td>
<td>3.49 (3.70–3.49)$^a$</td>
<td>3.41 (3.62–3.41)$^a$</td>
<td>3.30 (3.50–3.30)$^a$</td>
</tr>
<tr>
<td>$R_{	ext{merge}}$ or $R_{	ext{sym}}$</td>
<td>23.2 (8.52)$^a$</td>
<td>24.0 (8.20)$^a$</td>
<td>23.2 (8.52)$^a$</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.4 (97.7)$^a$</td>
<td>98.9 (96.6)$^a$</td>
<td>99.4 (97.7)$^a$</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.5 (7.1)$^a$</td>
<td>6.6 (6.3)$^a$</td>
<td>7.5 (7.1)$^a$</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td>1.2146</td>
<td>0.9000</td>
</tr>
<tr>
<td>Temperature (K)</td>
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<td>100</td>
<td>100</td>
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*Highest resolution shell is shown in parentheses.*

**TABLE 2**

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<th>Tantalum-3</th>
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<td>Ta$<em>{Br</em>{4}}$, remote</td>
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<tr>
<td>Space group</td>
<td>$P2_12_1$</td>
<td>$P2_12_1$</td>
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<tr>
<td>Cell dimensions: $a$, $b$, $c$ (Å)</td>
<td>210.6, 239.3, 300.4</td>
<td>210.6, 239.4, 300.5</td>
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<tr>
<td>Resolution (Å)</td>
<td>3.49 (3.70–3.49)$^a$</td>
<td>3.41 (3.62–3.41)$^a$</td>
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<tr>
<td>$R_{	ext{merge}}$ or $R_{	ext{sym}}$</td>
<td>23.2 (8.52)$^a$</td>
<td>24.0 (8.20)$^a$</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.4 (97.7)$^a$</td>
<td>98.9 (96.6)$^a$</td>
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<tr>
<td>Redundancy</td>
<td>7.5 (7.1)$^a$</td>
<td>6.6 (6.3)$^a$</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td>1.2146</td>
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<tr>
<td>Temperature (K)</td>
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*Highest resolution shell is shown in parentheses.*

**TABLE 3**

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<td>Bond angles (°) 0.984</td>
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<td>Outliers (%) 0.1</td>
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<td>Favored (%) 96.2</td>
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</table>
Crystal Structure of Eukaryotic Photosystem II

FIGURE 2. A part of the electron density map obtained from crystals of the red algal PSII tetramer at 2.76 Å resolution, showing the quality of the map and its fitting with the amino acid residues.

water molecules were found in each of the four monomers, among which one is associated with Mn4 as a terminal ligand, which corresponded to W1 in the cyanobacterial PSII structure, suggesting that this water molecule is rather well defined. Among the two chlorides found in the cyanobacterial PSII, only one was found which corresponded to Cl\(^{-}\) in the cyanobacterial structure (surrounded by backbone nitrogen D1-Asn\(^{338}\) and CP43-Glu\(^{153}\)) (5, 6, 33, 34). The Cl\(^{-}\) ion could not be assigned because of the low resolution. In addition to the calcium ion found in the Mn\(_4\)CaO\(_5\) cluster of OEC, another calcium ion was found in two monomers (Mol-1 and Mol-4) but absent in the other two monomers (Mol-2, Mol-3); it is located in one side end of the cylindrical structure of the PsbO subunit opposite to OEC and is surrounded by three residues corresponding to the cyanobacterial PsbO-Thr\(^{138}\), PsbO-Asn\(^{202}\), and PsbO-Val\(^{201}\). This calcium-binding site was also found in the native structure of the cyanobacterial PSII (5, 35), which is surrounded by several additional water molecules.

In addition to the protein chains, 35 chlorophylls, 9–10 \(\beta\)-carotenoids, 1 plastoquinone (Q\(_A\)), 1 non-heme iron, 1 bicarbonate, 12–20 lipids, and several unknown molecules were found in each monomer (Table 5). The electron density for the second bound quinone acceptor O\(_B\) was very weak because of the limited resolution.

Structure of the PsbQ Subunit and Its Association with PSII—
In the luminal side, additional electron densities were found underneath the luminal loop region of CP43 and close to PsbV but in the opposite side of PsbO. These electron densities were present in two of the four monomers (namely, Mol-2 and Mol-3) (Fig. 5), which do not have corresponding peptide chains in the cyanobacterial PSII structure (Fig. 3C). These electron densities were modeled as PsbQ\(_1\), which showed a four-helix bundle structure very similar to that of isolated higher plant and cyanobacterial PsbQ (Fig. 6) (36–39). No evidence for the presence of N-terminal lipid anchor was observed for the red algal PsbQ\(_1\), although it has been suggested to be present in Cyano-PsbQ. The absence of PsbQ\(_1\) in the other two monomers Mol-1 and Mol-4 is due to the effect of adjacent PSII dimer in the crystal packing. As depicted in Fig. 4, whereas one of the two PSII monomers in a dimer has a rather large space in the luminal region between the adjacent PSII dimer to accommodate the PsbQ\(_1\) protein, this part of the second monomer is in a close contact with another adjacent dimer, which may have affected the tight binding of the PsbQ\(_1\) protein in this region of the second monomer. This may result in a disordered location of PsbQ\(_1\) in this region of the crystals, although this subunit appeared to be retained in the crystal based on the SDS-PAGE analysis (Fig. 1).

As can be seen from Fig. 6, when the structure of the red algal PsbQ\(_1\) was superimposed with spinach PsbQ (Protein Data Bank code 1VYK) (37), the overall four-helix folding pattern was well conserved in the two structures. However, helices I and II were rotated against helices III and IV in the red algal PsbQ\(_1\) rather than those in the spinach PsbQ, resulting in an apparent shift of the two \(\alpha\)-helices by a width of approximately one \(\alpha\)-helix. In fact, the average root mean square deviation of the \(\alpha\)-helices I and II between the red algal PsbQ\(_1\) and spinach PsbQ (Protein Data Bank code 1VYK) (red alga: 111–142, 150–156; spinach: 47–78, 86–92) was 0.762 Å, which is apparently larger than the average root mean square deviation of 0.652 Å calculated for the helices III and IV (red alga: 170–214; spinach: 102–146) between the two proteins. This rotational structural difference may be related with the presence of a longer loop between \(\alpha\)-helices II and III in the red algal PsbQ\(_1\) than that of spinach PsbQ.

The structural differences between the red algal PsbQ\(_1\) and spinach PsbQ is likely to reflect the differences in the binding properties of these two subunits to PSII. In fact, a close examination on the interaction between PsbQ\(_1\) and the red algal PSII intrinsic proteins shows that a region in the middle of helix II was in close contact with a region (Pro\(^{191}\)–Asp\(^{195}\), PGGDG) in loop C from PsbC (CP43) (40). In particular, the regions around PsbQ\(_1\)-Arg\(^{150}\) and CP43-Pro\(^{191}\)–Asp\(^{195}\) form an interface with apparently opposite charges on the two surfaces from the two subunits (Fig. 6C), suggesting their possible association through electrostatic interactions. Importantly, the region surrounding PsbQ\(_1\)–Arg\(^{150}\) was highly conserved (Fig. 7), and both
PsbQ-H11032-Arg150 (Fig. 7) and the PGGGD motif (Pro191–Asp195) in the loop C of PsbC (Fig. 8) are completely conserved throughout the organisms whose sequences are available. Thus, it is clear that PsbQ-H11032-Arg150 and CP43-Pro191–Asp195 form an interaction site for the binding of PsbQ-H11032 to PSII, and to accommodate the binding counterpart from the loop C of the PsbC subunit, helix II of PsbQ-H11032 was forced to kink toward helix I. In turn, helix I had to kink away relative to the position of spinach PsbQ, leading to the apparent differences between the structures of red algal PsbQ-H11032 and spinach PsbQ. This further suggests that the region responsible for the association with PSII intrinsic subunits is different between the red algal PsbQ-H11032 and spinach PsbQ. Alternatively, there is another possibility that, because the structure of spinach PsbQ was determined in its free form (not bound to PSII), structural changes may have occurred upon its release from spinach PSII. Because the structure of PsbQ in its bound form to PSII has not been determined, whether such a structural change indeed occurred has to be clarified in future studies.

In addition to the interaction between PsbQ-H11032 and CP43 in the region described above, Lys132–Ser134 in the loop region connecting helix I and II of PsbQ-H11032 is located close to PsbO-Gln4 in the N-terminal region of PsbO, suggesting their possible interactions. In addition, the surface of PsbQ-H11032 is rich in positive charges (such as Arg139, Arg143, Lys155, etc.; Fig. 6C); these positive charges may also be important for its association with PSII through electrostatic interactions with the surface of CP43 having opposite potentials (Fig. 6C). These results indicate that PsbQ-H11032 associates with PSII mainly through its interactions with CP43, with possible additional contributions from its interaction with the N-terminal region of PsbO, which are in well agreement with the extrinsic nature of this subunit.

Two Additional Transmembrane Helices—The electron density map of the red algal PSII showed two additional transmembrane regions that do not have corresponding protein subunits in the cyanobacterial PSII (Fig. 5). These densities were found in PsbQ-H11032-Arg150 (Fig. 7) and the PGGGD motif (Pro191–Asp195) in the loop C of PsbC (Fig. 8) are completely conserved throughout the organisms whose sequences are available. Thus, it is clear that PsbQ-H11032-Arg150 and CP43-Pro191–Asp195 form an interaction site for the binding of PsbQ-H11032 to PSII, and to accommodate the binding counterpart from the loop C of the PsbC subunit, helix II of PsbQ-H11032 was forced to kink toward helix I. In turn, helix I had to kink away relative to the position of spinach PsbQ, leading to the apparent differences between the structures of red algal PsbQ-H11032 and spinach PsbQ. This further suggests that the region responsible for the association with PSII intrinsic subunits is different between the red algal PsbQ-H11032 and spinach PsbQ. Alternatively, there is another possibility that, because the structure of spinach PsbQ was determined in its free form (not bound to PSII), structural changes may have occurred upon its release from spinach PSII. Because the structure of PsbQ in its bound form to PSII has not been determined, whether such a structural change indeed occurred has to be clarified in future studies.

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Two Additional Transmembrane Helices—The electron density map of the red algal PSII showed two additional transmembrane regions that do not have corresponding protein subunits in the cyanobacterial PSII (Fig. 5). These densities were found in
all of the four monomers of the tetramer. Based on their electron density distributions, they were assigned to two new transmembrane helices. Because their sequences are not known at present, most of their residues were assigned as alanines, but some of the residues that have large electron densities protruded from the main chain were fitted with respective amino acid residues that best fit with the densities.

The two new helices were located in the peripheral region of a monomer close to the interface between two monomers in a dimer. Among the two new transmembrane helices, one is located outside of PsbI and close to the N-terminal region of D1 subunit (PsbA) in the stromal side (Fig. 9A); this subunit is designated S (chain ID: S). This helix was also close to a short helix formed by residues from Ser258 to Gly268 of CP43 in the stromal side. The other helix is designated W (chain ID: W), and is adjacent to the transmembrane helix II of CP47 (PsbB) and also close to the N-terminal region of PsbH in the stromal side (Fig. 9B).

Discussion

Red alga is the most primitive eukaryotic algae with their photosynthetic systems resemble in part the prokaryotic cyanobacteria and in part the eukaryotic algae, because the PSII of red algae is associated by phycobilisomes as light-harvesting antenna pigments, whereas its photosystem I binds light-harvesting complex I similar to those found in green algae and higher plant photosystem I (42). So far the structure of PSII has been solved from cyanobacteria at an atomic resolution (5, 6), whereas no crystal structure of eukaryotic PSII has been reported. The present study represents the first crystal structure analysis of eukaryotic PSII, which existed in a tetramer in a

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**TABLE 5**

Number of cofactors in each monomer of the tetrameric PSII in the crystal

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**FIGURE 4.** A part of unit cell in the crystals of red algal PSII, showing the contacts among adjacent PSII dimers. A, view perpendicular to the membrane plane along with a PSII dimer in the middle. The circled areas represent PsbQ', which is present in one side but absent in the opposite side of a dimer. B, same as A, but rotated by 180° along the membrane plane. PsbQ' was colored in red, and PsbZ was colored in green.

**FIGURE 5.** Structure of red algal PSII dimer (Mol-1, right; Mol-2, left) and the location of PsbQ' and two novel transmembrane helices. A, side view of the thylakoid membrane. The cycle with red dashed line represents the region where the PsbQ' subunit was missing. B, top view from the stromal side of the membrane. The electron densities for PsbQ' and the two novel helices (chains S and W) were depicted in red as omit maps contoured at 2σ in both A and B.
crystallographic asymmetric unit with an overall molecular mass of ~1,400 kDa (350 kDa × 4). Although the overall structure of the red algal PSII was found to be similar to that of cyanobacterial PSII, some unique and important new features were found. In the following text, we discuss these new features of the red algal PSII in relation to the evolution and function of PSII from cyanobacteria to higher plants.

One of the distinctive features of the red algal PSII is that it contains four extrinsic proteins, including the three subunits found in cyanobacterial PSII and the fourth subunit PsbQ with a low homology to the PsbQ protein of green algae and higher plants (11, 13, 15–22). Although the structures of purified PsbQ, as well as cyano-PsbQ, have been solved from higher plants and cyanobacteria (36–39), their association pattern with PSII was not clear so far. The structure of the red algal PSII contained PsbQ' in two monomers of the tetramer within the crystal, which allowed us to analyze its structure and interaction sites with PSII.

The location of PsbQ' was found to be underneath CP43 in the luminal side, which is in the opposite side of the major domain of PsbO and occupies a peripheral, outside region of the PSII dimer in the luminal surface. This location makes the four extrinsic proteins PsbO, PsbQ', PsbU, and PsbV forming a circle in the luminal surface, the middle of which is occupied by extrinsic loops of CP43 and D1 protruded from the membrane, which are involved in the binding and stabilization of the Mn₄CaO₅ cluster, the catalytic center for water splitting. Thus, one important function of the PsbQ' subunit is to provide a cap.
for the Mn₄CaO₅ cluster to prevent unfavorable attack from molecules such as reductants or chelating reagents from the lumenal bulk solution.

The location of PsbQ/H11032 revealed in the present study is consistent with the site suggested from single particle electron microscopic analysis of PSII from another red alga, *C. merolae* (43), although a previous microscopic analysis suggested a possibly different location of the PsbQ/H11032 in the red alga at a lower resolution (42). Very recently, however, Liu et al. (44) reported that cyano-PsbQ was located in the middle of the PSII dimer between two PsbO subunits from two monomers in the lumenal surface of cyanobacterial PSII and interacts with CP47 instead of CP43, based on the cross-linking of a PsbQ dimer and subsequent mass spectrometric analysis. A similar cross-linked dimer of PsbQ was observed with higher plant PSII, suggesting a similar arrangement of the PsbQ in the higher plant PSII (45). This is different from the site of PsbQ revealed by the present crystallographic studies, as well as the single particle image analysis of the red algal PSII (43). This discrepancy may reflect a different location of the homologous PsbQ protein in the different organisms examined, i.e. prokaryotic cyanobacteria versus eukaryotic red algae. Importantly, the interaction of PsbQ with CP43 has also been confirmed in higher plant PSII by cross-linking studies (46), which is consistent with the location of PsbQ we observed in the red algal PSII. In addition, Mummadisetty et al. (45) suggested that the observation of cross-

For the Mn₄CaO₅ cluster to prevent unfavorable attack from molecules such as reductants or chelating reagents from the lumenal bulk solution.

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linked PsbQ dimers in higher plant PSII can also be explained by cross-linking of the anti-parallel PsbQ bridging two different adjacent PSII dimers without restraining the location of this subunit in the interface region of the two monomers. This is consistent with the location of this subunit revealed in the present study and may indicate a different location of PsbQ between prokaryotic and eukaryotic PSII.

The overall structure of PsbQ is very similar to that of isolated higher plant and cyanobacterial PsbQ in that both have a four-helix folding pattern. However, helices I and II were bent in the middle; the reason for this bending appeared to be the interaction between a region in the middle of helix II of PsbQ and the motif PGGGD (Pro191–Asp195) in the loop C of CP43, in particular between PsbQ’-Arg150 (Arg78 of the mature protein) and the PGGGD motif of CP43 (Fig. 6), because they provided opposite surface potentials that may facilitate their association through electrostatic interactions. Both PsbQ’-Arg150 and Pro191–Asp195 of CP43 are conserved from sequences currently available (Figs. 7 and 8), suggesting their importance for the binding of PsbQ to PSII. These interactions explain why PsbQ alone can bind to PSII to a large extent in the red algal PSII as revealed from previous biochemical reconstitution studies (13). In addition, the loop region connecting helix I and II of PsbQ was located close to the N-terminal part of PsbO protruded from its cylindrical, β-barrel major domain, which may account for the fact that binding of PsbQ facilitates the efficient association of PsbO with PSII, a unique phenomenon found in the red algal PSII (13).

One important finding of the present study is that there are two additional transmembrane helices in the PSII monomer from the red alga, which have no corresponding peptides in the cyanobacterial PSII crystal structure (5, 6). To identify the origin of these two new subunits, we determined the subunit composition of the purified red algal PSII by extensive mass spectrometric analysis. As shown in supplemental Table S1, all of the known PSII transmembrane subunits were identified in the mass spectrometric data, among which, PsbW, a subunit known to be present in eukaryotic organisms but absent in prokaryotic genomes (8, 47) was also found. This subunit was also detected in the PSII purified from the red alga C. merolae recently (43). Thus, one of the two transmembrane helices revealed in the present study can be attributed to PsbW. It is interesting to note that the PsbW subunit has been suggested to function in the interaction between PSII core and light-harvesting antenna proteins LHClII in higher plants, because inactivation of this gene inhibited formation of the PSII-LHCII supercomplex (8, 48, 49). In red algae, however, there is no LHClII, and the light-harvesting function was fulfilled by phycobilisomes similar to that seen in cyanobacteria. Thus, the red algal PsbW subunit may serve a function different from that found in the green algal and higher plant PSII.

The identity of the remaining one novel helix is not clear; it could be PsbY, which was not observed in the crystal structure but identified by the mass spectrometry (supplemental Table S1). If this is the case, the position of PsbY must be different between cyanobacteria and eukaryotic PSII. The other possibility is that it may correspond to part of Psb27, which is also detected by the mass analysis (supplemental Table S1).

Although Psb27 is largely hydrophilic, it may contain a short hydrophobic domain to anchor it to the membrane. Finally, it may represent a yet unknown subunit in the red algal PSII. In addition to the above two novel transmembrane helices, all of the transmembrane subunits found in the cyanobacterial PSII were present in the red algal PSII, implying that the eukaryotic PSII attained additional complexity during evolution from its ancestor cyanobacterial PSII.

Author Contributions—J.-R. S. planned and organized the experiments and supervised the purification and crystallization. H. Adachi purified and crystallized PSII from C. caldarium with the help of K. K. and I. E.; and H. Adachi, Y. U., T. T., K. K., J.-R. S. collected the X-ray diffraction data; and Y. U., T. T., H. Adachi, and N. K. analyzed the diffraction data. H. Ago analyzed the structure with the help of M. M.; L. T., G. H., and T. K. purified PSII from C. merolae; and Z. L., F. W., and H. Z. performed the MS analysis. H. Ago and J.-R. S. wrote the manuscript with the input from all of the authors.

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References


