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Binding and functional properties of five extrinsic proteins in oxygen-evolving Photosystem II from a marine centric diatom, *Chaetoceros gracilis*

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Oxygen-evolving Photosystem II (PSII) isolated from a marine centric diatom, *Chaetoceros gracilis*, contains a novel extrinsic protein (Psb31) in addition to four red algal-type extrinsic proteins of PsbO, PsbQ', PsbV and PsbU. In this study, the five extrinsic proteins were purified from alkaline Tris-extracts of the diatom PSII by anion and cation exchange chromatographic columns at different pHs. Reconstitution experiments in various combinations with the purified extrinsic proteins showed that PsbO, PsbQ' and Psb31 rebound directly to PSII in the absence of other extrinsic proteins, indicating that these extrinsic proteins have their own binding sites in PSII intrinsic proteins. On the other hand, PsbV and PsbU scarcely rebound to PSII alone and their effective bindings required the presence of all of the other extrinsic proteins. Interestingly, PSII reconstituted with Psb31 alone considerably restored the oxygen-evolving activity in the absence of PsbO, indicating that Psb31 serves as a substitute in part for PsbO in supporting oxygen evolution. A significant difference found between PSIIs reconstituted with Psb31 and with PsbO is that the oxygen-evolving activity of the former is scarcely stimulated by Cl^- and Ca^{2+} ions but that of the latter is largely stimulated by these ions, although rebinding of PsbV and PsbU activated oxygen evolution in the absence of Cl^- and Ca^{2+} ions in both the former and latter PSIIs. Based on these

results, we proposed a model for the association of the five extrinsic proteins with intrinsic proteins in diatom PSII and compared it with those in PSIIs from the other organisms.

Oxygen-evolving Photosystem II (PSII) is a thylakoid membrane-located, multi-subunit pigment complex catalyzing light-induced electron transfer from water to plastoquinone, with the concomitant production of molecular oxygen. The PSII complex consists of a number of intrinsic proteins and several extrinsic proteins associated with the lumenal side. So far PSII membrane fragments and core complexes that are highly active in oxygen evolution and retain all of the extrinsic proteins have been isolated from cyanobacteria (1-3), red alga (4, 5), *Euglena* (6), green alga (7) and higher plants (8-10). Among these PSII complexes from a wide variety of organisms, the major intrinsic core proteins are largely conserved, whereas the extrinsic proteins are significantly different among different plant species.

The differences in the composition of the extrinsic proteins and their binding patterns in PSII from various organisms is illustrated in the schematic models shown in Fig. 1. The direct and indirect association of various extrinsic proteins at the lumenal side are based on various reconstitution and cross-reconstitution studies reported so far (for a recent review, see 11). Purified cyanobacterial PSII (Fig. 1A) contains three extrinsic proteins of PsbO, PsbV and PsbU, in which PsbO and PsbV can directly bind to PSII intrinsic proteins essentially independent of the presence of other proteins, whereas the binding of PsbU requires the presence of both PsbO and PsbV (1-3, 12, 13). In red algal PSII (Fig. 1B), a fourth extrinsic protein, the unique 20 kDa protein (PsbQ') is present in addition to the three cyanobacterial extrinsic proteins (5, 14). Among the four extrinsic proteins, PsbO and PsbQ' can directly bind to PSII intrinsic proteins essentially independent of the presence of other proteins, whereas the effective binding of PsbV and PsbU requires the presence of both PsbO and PsbQ' (5). These results suggested that the binding property of PsbV is different between cyanobacterial and red algal PSIIs in that the former binds directly to PSII but the latter binds to PSII through its interaction with the other three extrinsic proteins. This difference of the binding property was shown to depend mainly on the structure of PSII intrinsic proteins but not that of PsbV by cross-reconstitution experiments using PsbV and PSII from the two organisms (15).

In contrast to cyanobacterial and red algal PSII, PSIIs of *Euglena*, green algae and higher plants contain PsbP and PsbQ proteins instead of PsbV and PsbU (6-10). In green algal and *Euglena* PSIIs (Fig. 1D), PsbP and PsbQ as well as PsbO can directly bind to PSII independent of the other extrinsic proteins, and PsbO functionally re-bind to PSII which has been reconstituted with PsbP

and PsbQ (6, 7), indicating that each of the three extrinsic proteins has their own binding sites in PSII which are independent of the other extrinsic proteins (7). Higher plant PSII also contains PsbO, PsbP, PsbQ as extrinsic proteins. However, their binding properties are largely different from those of the corresponding proteins in PSII of *Euglena* and green algae. In higher plant PSII, only PsbO can directly bind to PSII, whereas PsbP cannot bind to PSII directly and associates with PSII only through its interaction with PsbO, and PsbQ functionally associates with PSII only through its interaction with both PsbO and PsbP (16). Detailed analysis on the differences of the binding properties between green algal and higher plant PSIIs has been performed by cross-reconstitution experiments using extrinsic proteins and PSII from the two organisms (17). The results showed that the green algal PsbP and PsbQ proteins can directly bind to green algal PSII but not to higher plant PSII, in the absence of PsbO, whereas the higher plant PsbP and PsbQ proteins cannot bind to both higher plant and green algal PSIIs in the absence of PsbO, and functionally bind to higher plant PSII only in the presence of higher plant PsbO but not in the presence of green algal PsbO (17).

Among the variously different extrinsic proteins, PsbO is present in all of the oxygenic photosynthetic organisms and plays an important role in maintaining the stability and activity of the Mn_4Ca-cluster (11, 23, 24). On the other hand, the PsbV and PsbU proteins in cyanobacterial and red algal PSIIs or the PsbP and PsbQ proteins in green algal and higher plant PSIIs function to optimize the availability of Ca^{2+} and Cl^- cofactors for water oxidation (1, 4-7, 12, 25-27). The PsbQ' protein in red algal PSII is not involved directly in oxygen evolution but required for effective binding of the PsbV and PsbU proteins (5). These facts imply that PsbV and PsbU were replaced by PsbP and PsbQ during evolution from prokaryotic cyanobacteria and the primitive eukaryotic red algae to the green lineage *Euglena*, green algae and higher plants, and PsbQ' may be an intermediate between the PsbQ-like proteins in cyanobacteria and the mature PsbQ protein in higher plants. The distribution of these extrinsic proteins in various organisms was examined using a variety of antibodies, which showed that they have been diverged into cyanobacterial-type (PsbO, PsbV and PsbU), red algal-type (PsbO, PsbQ', PsbV and PsbU) and green algal-type (PsbO, PsbP and PsbQ) during early phases of evolution after a primary endosymbiosis (28).

It should be pointed out that PsbP-like and PsbQ-like proteins have been found in cyanobacterial thylakoid membranes and purified PSII (3). The binding characteristics and functions of these proteins are different from those of the extrinsic PsbP, PsbQ and PsbQ' proteins (see Review (11)), and they have been suggested to be lipoproteins and to play regulatory roles in maintaining the PSII activity in nutrient-limiting media depleted of Cl^-, Ca^{2+} or iron in the prokaryotic

cyanobacteria (18, 19). The major function of PsbQ-like protein was shown to stabilize PsbV, thereby contributing to the protection of the catalytic Mn_4Ca-Cl-cluster of the water oxidation machinery (20). The specific binding of PsbQ-like protein to cyanobacterial PSII was also suggested based on the co-purification of PSII with a His-tagged PsbQ-like protein (21). The binding properties and functions of some other hydrophilic, peripheral PSII subunits including PsbP-like and PsbQ-like proteins, Psb27 and PsbR has been reviewed by Roose et al. (22).

Recently, we succeeded for the first time in preparation of PSII retaining a high oxygen-evolving activity from a marine centric diatom, *Chaetoceros gracilis* (29, 30). The diatom PSII was found to contain a novel, fifth extrinsic protein (referred to as Psb31 following the nomenclature for PSII subunits (3)) in addition to the four red algal-type extrinsic proteins of PsbO, PsbQ', PsbV and PsbU (Fig. 1C) (29, 30). The gene encoding the novel Psb31 protein was cloned and sequenced (31), which showed that the deduced protein contained three characteristic leader sequences targeted for chloroplast endoplasmic reticulum membrane, chloroplast envelope membrane and thylakoid membrane, indicating that Psb31 is encoded in the nuclear genome and constitutes one of the extrinsic proteins located on the lumenal side (Fig. 1C) (31). The function of the Psb31 protein, however, remains to be clarified. For understanding of the oxygen-evolving complex in diatom PSII, it is important to elucidate the binding and functional properties of the novel Psb31 protein. In this study, the five extrinsic proteins were purified from the diatom PSII, and reconstitution experiments using the purified extrinsic proteins in various combinations with PSII were performed to examine the binding and functional properties of the five extrinsic proteins, especially the Psb31 protein.

EXPERIMENTAL PROCEDURES

Preparation of oxygen-evolving PSII from a marine centric diatom, Chaetoceros gracilis - A marine centric diatom, *C. gracilis*, was grown in artificial seawater as described previously (29). Thylakoid membranes and oxygen-evolving PSII were prepared according to Nagao et al. (29, 30). The cells suspended in a medium containing 1 M betaine, 50 mM MES-NaOH (pH 6.5) and 5 mM $MgCl_2$ were disrupted by freeze-thawing and then incubated in the presence of DNase I and 1 mM PMSF for 30 min at 0ºC in the dark. The supernatants after centrifugation at 3,000 x g for 3 min were centrifuged at 40,000 x g for 10 min and its precipitates (thylakoid membranes) were suspended in a medium containing 1 M betaine, 50 mM MES-NaOH (pH 6.5) and 1 mM EDTA (buffer A). The thylakoid membranes were treated with 1% Triton X-100 in buffer A at 1 mg chlorophyll (Chl)/ml for 5 min at 0ºC in the dark and then fractionated by differential centrifugations according to (29). The

resulting oxygen-evolving PSII particles (crude PSII) contained a large amount of fucoxanthin chlorophyll *a*/*c*-binding protein (FCP), and were remarkably unstable, as a significant inactivation of oxygen evolution, Chl bleaching and degradation of PSII subunits were observed during incubation at 25ºC in the dark (30). The crude PSII (1 mg Chl/ml) suspended in buffer A was then immediately solubilized with 1% Triton X-100 for 20 min at 0ºC in the dark and applied to a DEAE-Toyopearl 650M column equilibrated with the buffer A containing 0.03% Triton X-100. After the column was washed with 2-3 bed volumes of the equilibrating buffer, a green fraction (purified PSII lacking major components of FCP) was eluted at 180 mM NaCl according to (30). The purified PSII was concentrated by centrifugation at 40,000 x g for 20 min after addition of 10% polyethylene glycol 6,000 and suspended in a medium containing 0.4 M sucrose and 40 mM MES-NaOH (pH 6.5) (buffer B) and stored at −196ºC. The purified PSII lacking major components of FCP was much stable compared with the crude PSII, as the inactivation of oxygen evolution, Chl bleaching and degradation of PSII subunits observed in the crude PSII were largely suppressed in the purified PSII (30). Thus, the purified PSII was used for release-reconstitution experiments in this study.

Chl concentrations were determined in 90% acetone using the equation of Jeffrey and Humphrey (32).

Purification procedures of five extrinsic proteins - The crude PSII suspended in 10 mM MES-NaOH (pH 6.5) (buffer C) was treated with 1 M Tris-HCl (pH 8.5) at 0.5 mg Chl/ml for 1 h on ice in the dark, to release all of the extrinsic proteins of PsbO, PsbQ', PsbV, Psb31 and PsbU (29, 30, 33). The treated samples were centrifuged at 227,000 x g for 30 min after addition of 14% polyethylene glycol 1,450. The supernatants were dialyzed using Spectra/Por Dialysis Membrane (MWCO:8,000) (Spectrum Laboratories) against buffer C after addition of 1 mM PMSF, 1 mM EDTA and 10 μl Benzamidine Sepharose 6B (GE Healthcare, UK) to prevent cleavages of the extrinsic proteins by endogenous proteases. The dialyzed samples were applied to a DEAE-Toyopearl 650M column (pH 6.5) equilibrated with buffer C and then the fraction containing PsbO and PsbV was eluted at 0.2 M NaCl. The fraction passed through the DEAE column was applied to a CM-Toyopearl 650M column (pH 6.5) equilibrated with buffer C and then Psb31 was eluted at 0.2 M NaCl. The fraction passed through the CM column (pH 6.5) was dialyzed against 10 mM citric acid-NaOH (pH 5.5) and applied to another CM-Toyopearl 650M column (pH 5.5) equilibrated with the same buffer. PsbQ' was eluted at 0.1 M NaCl. The fraction passed through the CM column was dialyzed against 10 mM citric acid-NaOH (pH 4) and applied to a CM-Toyopearl 650M column (pH 4) equilibrated with the same buffer.

PsbU was eluted at 1 M NaCl. The fraction containing PsbO and PsbV eluted at 0.2 M NaCl from the DEAE-Toyopearl 650M column (pH 6.5) was dialyzed against 10 mM citric acid-NaOH (pH 5.5) and applied to a CM-Toyopearl 650M column (pH 5.5) equilibrated with the same buffer. PsbV was passed through the column and PsbO was eluted at 1 M NaCl. A flow chart for the purification procedure of the five extrinsic proteins from the crude PSII is shown in Supplemental Fig. S1.

The purified extrinsic proteins were concentrated by a small size of each column and/or ultrafiltration, and then dialyzed against 10 mM MES-NaOH (pH 6.5). The concentrations of purified extrinsic proteins were determined by a BCA Protein Assay Kit (PIERCE) (34).

Dissociation of extrinsic proteins - The purified PSII was treated either with 1 M NaCl, 1 M $MgCl_2$, 1 M Tris-HCl (pH 8.5), 2.6 M urea plus 0.2 M NaCl, or 4 M urea plus 0.2 M NaCl, at 0.25 mg Chl/ml for 20 min on ice in the dark. The treated samples were centrifuged at 40,000 x g for 10 min after addition of 10% polyethylene glycol 6,000. The polypeptides of the resulting precipitates and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE - Samples were solubilized with 5% lithium lauryl sulfate and 75 mM dithiothreitol for 30 min at room temperature. The solubilized samples (1 μg Chl) were applied to a gradient gel containing 16–22% acrylamide and 7.5 M urea according to Ikeuchi and Inoue (35). After electrophoresis at a constant current of 8 mA for 15 h, gels were stained with Coomassie Brilliant Blue R-250 (CBB) and photographed.

Reconstitution experiments - For reconstitution, the purified PSII was treated with 4 M urea/0.2 M NaCl to remove all of the extrinsic proteins. The urea/NaCl-treated PSII was incubated with the extrinsic proteins in various combinations for 20 min on ice at 0.05 mg Chl/ml. The extrinsic protein-PSII ratio used was 3 : 1 during reconstitution, based on the estimation that each PSII reaction center contains 42 molecules of Chl *a* (30) The reconstitution experiments were performed in buffer B containing 10 μM DCIP, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 10 mM $CaCl_2$ on ice at room light (7-8 μmol photons/m^2/s) (Mn-photoactivation condition (36)). After reconstitution, the mixtures were centrifuged at 40,000 x g for 5 min after addition of 10% polyethylene glycol 6,000, and the resulting precipitates were suspended in buffer B and used for electrophoretic analysis and oxygen evolution measurements. Unless otherwise described, the amount of each extrinsic proteins rebound was determined by scanning the CBB-stained gel and quantification of the peak area corresponding to each band with the NIH image software (ImageJ)

(http://rsb.info.nih.gov/ij/). The amount of each extrinsic protein in untreated PSII was taken as 100%.

Immunological assays - The relative amounts of the PsbV and PsbU proteins rebound by reconstitution experiments were estimated by immunological assays with antibodies raised against PsbV from a red alga, *Cyanidium caldarium* (28), and against PsbU from the diatom, *C. gracilis*. For immunological assays, proteins on the SDS-PAGE gel were transferred onto a polyvinylidene fluoride (PVDF) membrane, reacted with respective antibodies, and then Horseradish peroxidase (HRP)-conjugated anti-IgG was used as a secondary antibody. Chemiluminescent detection of HRP was carried out using ECL Western Blotting Detection Reagents (GE Healthcare, UK) and an ECL camera system using FP300B film (Fuji film). The peak area corresponding to each band was calculated with the NIH image software (ImageJ) (http://rsb.info.nih.gov/ij/).

Assay of oxygen-evolving activity - Oxygen evolution was measured with a Clark-type electrode at 25ºC in buffer B with 0.4 mM phenyl-*p*-benzoquinone (PBQ) as the electron acceptor in the absence or presence of 10 mM NaCl or 5 mM $CaCl_2$.

RESULTS

Purification of PsbO, PsbQ', PsbV, Psb31 and PsbU - As shown in Fig. 2, the five extrinsic proteins (lane 2) were released from the crude PSII (lane 1) by alkaline Tris-treatment, and then purified from the Tris-extracts by anion and cation exchange chromatographic columns at different pHs (see Supplemental Fig. S1). The procedures we used yielded highly purified PsbO (lane 3), PsbQ' (lane 4), PsbV (lane 5), Psb31 (lane 6), and PsbU (lane 7), which were identified by immunoblotting and N-terminal sequencing analysis according to the methods described in our previous papers (29, 30). However, a small amount of the large subunit (L) of riburose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) was contaminated in the fraction of PsbV (lane 5), which will not interfere with our reconstitution experiments.

Dissociation of extrinsic proteins by various treatments - The extrinsic proteins of PSII have been reported to be released by various treatments. In spinach PSII, the PsbP and PsbQ proteins are selectively released with 1 M NaCl-wash (37), and all of the three extrinsic proteins of PsbO, PsbP and PsbQ can be released with 1 M $CaCl_2$- (38), 2.6 M urea plus 0.2 M NaCl- (39), or 1 M Tris (pH 8.5)-treatment (33). In red algal PSII, no extrinsic proteins are released by 1 M NaCl-wash, whereas treatments with 1 M $CaCl_2$, 2.6 M urea plus 0.2 M NaCl, or 1 M Tris (pH 8.5) released all of the extrinsic proteins (4, 5). In green algal PSII, the PsbP and PsbQ proteins are only partially released by 1 M NaCl-wash and most of the three extrinsic proteins are released by treatments with 2.6 M urea plus 0.2 M NaCl,

1 M Tris (pH 8.5), or 1 M $CaCl_2$, although a small amount of the PsbO protein remains bound after these treatments (7). These indicate that the binding properties of the extrinsic proteins are different among different organisms.

Thus, we first examined the release of the five extrinsic proteins in diatom PSII by various treatments. For release-reconstitution experiments, we used the purified PSII because the purified PSII was much stable compared with the crude PSII as described above. Fig. 3 shows dissociation of the extrinsic proteins from the purified PSII by various treatments. The PsbO, PsbQ', PsbV and Psb31 proteins were only partially released together with the large (L) and small (S) subunits of RubisCO by 1 M NaCl-wash (lane 2). Treatments with 1 M $MgCl_2$ (lane 3), 1 M Tris (pH 8.5) (lane 4), or 2.6 M urea plus 0.2 M NaCl (lane 5) completely released the PsbQ', Psb31 and PsbU proteins, but a considerable amount of the PsbO and PsbV proteins remained bound to PSII after these treatments. When the purified PSII was treated with 4 M urea plus 0.2 M NaCl, most of the PsbO and PsbV proteins were released together with a complete release of the other extrinsic proteins, although a trace amount of PsbO remained bound (lane 6). We attempted to remove the PsbO protein completely by various treatments, and found that the protein could be completely released by twice treatments with 4 M urea plus 0.2 M NaCl. However, the PSII treated twice with 4 M urea plus 0.2 M NaCl showed a very low oxygen-evolving activity even after complete rebinding of all of the extrinsic proteins, suggesting that the twice treatments may have damaged the Mn_4Ca-cluster itself. Thus, the purified PSII treated once with 4 M urea plus 0.2 M NaCl was used as the extrinsic proteins-deleted PSII for reconstitution experiments in this study.

Restoration of oxygen evolution by reconstitution with all of the extrinsic proteins under normal or Mn-photoactivation conditions- Table 1 shows restoration of oxygen evolution by reconstitution with all of the extrinsic proteins of PsbO, PsbQ', PsbV, Psb31 and PsbU to the extrinsic proteins-deleted PSII prepared by 4 M urea plus 0.2 M NaCl-treatment. When the reconstitution experiments were performed in a medium containing 0.4 M sucrose and 40 mM MES-NaOH (pH 6.5) (buffer B) on ice in the dark (normal condition), the restoration of oxygen evolution was 20-23% of the original activity in the absence and presence of Cl^- and Ca^{2+} ions, respectively. This level of restoration is considerably lower than those observed with PSIIs from other organisms. For example, upon reconstitution of all of the extrinsic proteins under normal condition, restoration of the oxygen evolution has been reported to be 42% in spinach PSII (12), 42% in green algal PSII (7), 40% in *Euglena* PSII (6), 59-74% in red algal PSII (5, 12), and 79% - 87% in PSII from a thermophilic cyanobacterium (1, 12). This may partly be

due to the harsh treatment of 4 M urea plus 0.2 M NaCl required to release all of the extrinsic proteins from the diatom PSII, which may have damaged the Mn_4Ca-cluster to some extent. In order to obtain a higher level of restoration of the oxygen evolution, we screened various conditions for reconstitution, and found that the oxygen evolution restored to 46-50% of the original activity when reconstitution experiments were performed in buffer B containing 10 μM DCIP, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 10 mM $CaCl_2$ on ice at room light (7-8 μmol photons/m^2/s) (Table 1), which is comparable to the restoration level observed in spinach, green algal, *Euglena* and red algal PSIIs. This reconstitution condition is the same as that used for Mn-photoactivation (36). Thus, in this study, we performed reconstitution experiments under the Mn-photoactivation condition in order to achieve the maximum binding of extrinsic proteins and restoration of oxygen evolution.

Binding properties of the five extrinsic proteins - To examine which proteins in the five extrinsic proteins of diatom PSII directly interact with PSII intrinsic proteins, each of the extrinsic proteins was separately reconstituted with the extrinsic proteins-deleted PSII. The results obtained are shown in Fig. 4. As described above, treatment of the purified PSII (lane 1) with 4 M urea plus 0.2 M NaCl released most of the five extrinsic proteins (lane 2). Reconstitution with PsbO (lane 3), PsbQ' (lane 4) or Psb31 (lane 6) alone resulted in the binding of the respective proteins to the extrinsic proteins-depleted PSII to a level equal to those in the purified PSII, whereas reconstitution with PsbV (lane 5) or PsbU (lane 7) alone resulted in almost no re-binding of these two proteins. When all of the five extrinsic proteins were reconstituted together, the PsbV and PsbU proteins rebound, together with the other three proteins, to a level equal to those in the purified PSII (lane 8). It is noted that the band of PsbV became somewhat broad after reconstitution, although the reason for this is unknown at present. The broad band was confirmed to be PsbV but not the small subunit (S) of RubisCO by immunoblotting analysis, as shown in Supplemental Fig. S2. These results indicate that the PsbO, PsbQ' and Psb31 proteins can directly bind to PSII intrinsic proteins independent of the other extrinsic proteins, whereas the effective binding of the PsbV and PsbU proteins requires the presence of other extrinsic proteins.

In order to examine the extrinsic proteins that are required for the binding of PsbV and PsbU to PSII, we carried out reconstitution experiments with each of the other four proteins in combination with PsbV or PsbU. We first carried out the reconstitution experiments with various extrinsic proteins in combination with PsbV. Because the band of the small subunit (S) of RubisCO appeared close to the band of PsbV (Fig. 3) and the band of PsbV became broad after

reconstitution, the relative amounts of PsbV rebound were determined by immunoblotting assay with an antibody against PsbV (Supplemental Fig. S2A), and the results obtained were summarized in Table 2. The amount of the PsbV protein rebound in the presence of the PsbO, PsbQ' or Psb31 protein increased to 42%, 56% or 54% from 14% in the absence of other extrinsic proteins, respectively. When reconstitution was performed in combination with PsbO and PsbQ', PsbQ' and Psb31, or PsbO and Psb31, the amount of the PsbV protein rebound increased to 65%, 72% or 61%, respectively, which further increased to 84% when ~~the~~ reconstitution was performed in combination with the three extrinsic proteins of PsbO, PsbQ' and Psb31. When all of the five extrinsic proteins were reconstituted together, the amount of the PsbV protein rebound reached to 100%. These results indicate that the PsbV protein partially interacts with each of the PsbO, PsbQ', Psb31 and PsbU proteins, and its complete binding requires the presence of all of the other four extrinsic proteins. Alternatively, interactions among the other four proteins may be necessary to create an optimum site for the binding of PsbV.

Second, we carried out the reconstitution experiments with each of the other four proteins in combination with the PsbU protein. Because the band of the α subunit of cytochrome *b*559 (Cyt *b*559α) appeared close to the band of PsbU (Fig. 3), the relative amounts of PsbU rebound were determined by immunoblotting assay with an antibody against PsbU (Supplemental Fig. S2B), and the results obtained were summarized in Table 3. The amount of the PsbU protein rebound in the presence of the PsbO, PsbQ' or Psb31 proteins increased to 34%, 30% or 34% from 5% in the absence of the other extrinsic proteins, respectively. In the presence of PsbO and PsbQ', PsbQ' and Psb31, or PsbO and Psb31, the amount of the PsbU protein rebound increased to 41%, 54% or 66%, respectively, and in the presence of the three extrinsic proteins of PsbO, PsbQ' and Psb31, it increased to 78%. When all of the five extrinsic proteins were reconstituted together, the PsbU protein completely rebound. These results indicate that the PsbU protein also partially interacts with each of the PsbO, PsbQ', Psb31 and PsbV proteins, and its complete binding requires the presence of all of the other four extrinsic proteins. Here again, the possibility exists that interactions among the other four proteins may be necessary to create an optimum site for the binding of PsbU.

Third, we carried out the reconstitution experiments with each of the other three proteins in combination with both the PsbV and PsbU proteins, to examine the possible interaction between PsbV and PsbU. The result of immunoblotting assay obtained is shown in Supplemental Fig. S2C and summarized in Table 4. The amount of PsbV rebound significantly increased from 14% in the absence of PsbU (Table 2) to 56% in the presence of PsbU (Table 4), and that of

PsbU also increased from 5% in the absence of PsbV (Table 3) to 23% in the presence of PsbV (Table 4). This suggests that PsbV and PsbU only partially interact with PSII intrinsic proteins by themselves, and an interaction between them exists, which enhanced their direct binding to PSII significantly. When reconstitution was carried out together with PsbO, PsbQ' or Psb31, the amount of PsbV rebound increased from 42%, 56% or 54% in the absence of PsbU (Table 2) to 68%, 76% or 72% in the presence of PsbU (Table 4), respectively, and that of PsbU increased from 34%, 30% or 34% in the absence of PsbV (Table 3) to 55%, 52% or 61% in the presence of PsbV (Table 4), respectively. These results are in agreement with the existence of an interaction between PsbV and PsbU that facilitates binding of the respective proteins to PSII. All of the extrinsic proteins were, however, required for the complete binding of the PsbV and PsbU proteins (Tables 2-4). These suggest that the PsbV and PsbU proteins partially interact with all of the other extrinsic proteins, and the interaction between PsbV and PsbU is indispensible for their complete binding to PSII.

Functions of the five extrinsic proteins - Table 5 shows restoration of the oxygen-evolving activity upon reconstitution with the extrinsic proteins in various combinations. The extrinsic proteins-depleted PSII obtained by treatment with 4 M urea plus 0.2 M NaCl showed no oxygen evolution in the absence of Cl^- and Ca^{2+} ions. When the extrinsic proteins-depleted PSII was reconstituted with PsbO alone, the oxygen-evolving activity restored to 8% of the original activity in the absence of Cl^- and Ca^{2+} ions, to 24% in the presence of Cl^- ion and to 44% in the presence of both Cl^- and Ca^{2+} ions. The requirements for Cl^- and Ca^{2+} ions in the PSII reconstituted with PsbO in the absence of the other extrinsic proteins were similar to those in PSII of spinach (37, 38), a green alga (7), a red alga (5, 12) and a cyanobacterium (1, 2, 12). In the PSII reconstituted with PsbQ' alone, no restoration of the oxygen evolution was detected, indicating that the PsbQ' protein is not involved directly in oxygen evolution but required for effective binding of the PsbV and PsbU proteins (Tables 2-4), which are similar to those of red algal PSII (5). Interestingly, PSII reconstituted with Psb31 alone showed an oxygen-evolving activity of 128 μmol O_2/mg Chl/h in the absence of Cl^- and Ca^{2+} ions (6% of the original activity), but the activity was scarcely stimulated in the presence of Cl^- and Ca^{2+} ions, which will be described in detail later.

The PSII reconstituted with PsbO in combination with PsbQ', Psb31, or PsbQ' plus Psb31 showed similar activity to that of PSII reconstituted with PsbO alone, suggesting that there is no cooperative activation of oxygen evolution between PsbO and Psb31. On the other hand, the activity of PSII reconstituted with PsbO in

combination with the three extrinsic proteins of PsbQ’, Psb31 and PsbV was restored to 13% of the original activity in the absence of Cl^- and Ca^{2+} ions, to 36% in the presence of Cl^- ion and to 45% in the presence of Cl^- and Ca^{2+} ions. This indicates that rebinding of PsbV largely decreased the requirement for Ca^{2+} ion on oxygen evolution, suggesting that PsbV mainly functions to optimize the availability of the Ca^{2+} cofactor. In PSII reconstituted with all of the extrinsic proteins, the activity was restored to 46% of the original activity even in the absence of Cl^- and Ca^{2+} ions and scarcely stimulated in the presence of Cl^- and Ca^{2+} ions. These indicate that the PsbV and PsbU proteins function to optimize the availability of Cl^- and Ca^{2+} cofactors for water oxidation, with PsbV mainly functioning to optimize the availability of Ca^{2+} and PsbU mainly functioning to optimize the availability of Cl^-.

The observation that PSII reconstituted with Psb31 alone showed an oxygen-evolving activity of 128-156 μmol O_2/mg Chl/h even in the absence of PsbO (Table 5) is significant. To further examine the function of Psb31, we compared the restoration of oxygen-evolving activity in PSII reconstituted with Psb31 in combination with the other extrinsic proteins in the absence of PsbO, with that in PSII reconstituted with PsbO in combination with other proteins. The results obtained are shown in Table 6. PSII reconstituted with Psb31 together with PsbQ’ showed similar activity to that of PSII reconstituted with Psb31 alone. On the other hand, when PSII was reconstituted with Psb31 together with both of PsbQ’ and PsbV, the activity increased to 292 μmol O_2/mg Chl/h (13% recovery) in the presence of Cl^- and 320 μmol O_2/mg Chl/h (14% recovery) in the presence of both Cl^- and Ca^{2+} ions, whereas the activity in the absence of Cl^- and Ca^{2+} ions was scarcely increased. This indicates that when the PsbV protein was rebound to PSII reconstituted with Psb31, it functions to activate the oxygen evolution in the presence of Cl^- but in the absence of Ca^{2+} ion. Furthermore, the activity of PSII reconstituted with Psb31 together with the three extrinsic proteins of PsbQ’, PsbV and PsbU increased to 343 μmol O_2/mg Chl/h (16% recovery) in the absence of Cl^- and Ca^{2+} ions, to 452 μmol O_2/mg Chl/h (20% recovery) in the presence of Cl^- ion, and to 470 μmol O_2/mg Chl/h (21% recovery) in the presence of Cl^- and Ca^{2+} ions. These results indicate that when the PsbV and PsbU proteins were rebound to PSII reconstituted with Psb31, they were able to activate the oxygen evolution in the absence of Cl^- and Ca^{2+} ions.

On the other hand, PSII reconstituted with PsbO together with both of PsbQ’ and PsbV showed the oxygen-evolving activity of 257 μmol O_2/mg Chl/h (12% recovery) in the absence of Cl^- and Ca^{2+} ions, 672 μmol O_2/mg Chl/h (30% recovery) in the presence of Cl^- ion, and 1,075 μmol O_2/mg Chl/h (48% recovery) in the presence of Cl^- and Ca^{2+} ions (Table 6). Upon reconstitution with PsbO

together with the three extrinsic proteins of PsbQ', PsbV and PsbU, the activity reached to 711 μmol O_2/mg Chl/h (33% recovery) in the absence of Cl^- and Ca^{2+} ions, to 784 μmol O_2/mg Chl/h (35% recovery) in the presence of Cl^- ion, and to 1,081 μmol O_2/mg Chl/h (48% recovery) in the presence of Cl^- and Ca^{2+} ions (Table 6). Furthermore, PSII reconstituted with all of the extrinsic proteins showed 988 μmol O_2/mg Chl/h (46% recovery) in the absence of Cl^- and Ca^{2+} ions, 1,113 μmol O_2/mg Chl/h (50% recovery) in the presence of Cl^- ion, and 1,130 μmol O_2/mg Chl/h (50% recovery) in the presence of Cl^- and Ca^{2+} ions, as shown in Table 5.

These indicate that the function of PsbO on oxygen evolution can partially be replaced by the novel Psb31 protein in diatom PSII, and all of the extrinsic proteins including PsbO and Psb31 are required for the maximal oxygen-evolving activity.

DISCUSSION

Binding features of the five extrinsic proteins in diatom PSII - The present study demonstrated that, among the five extrinsic proteins of PSII from a marine centric diatom, *Chaetoceros gracilis*, the PsbO, PsbQ' and Psb31 proteins can separately bind to PSII independent of the other extrinsic proteins (Fig. 4). This indicates that these three extrinsic proteins have their own binding sites on PSII intrinsic proteins. On the other hand, the other two proteins, PsbV and PsbU, scarcely bound to PSII unless reconstituted in combinations with the other extrinsic proteins (Fig. 4). The complete binding of PsbV and PsbU to PSII required the presence of all of the other extrinsic proteins (Tables 2-4). On the basis of these results, we propose a simplified model for the association of the five extrinsic proteins with PSII intrinsic proteins (Fig. 1C). In this model, it is shown that the PsbO, PsbQ' and Psb31 proteins have direct contacts with PSII intrinsic proteins, whereas the PsbV and PsbU proteins mainly associate with PSII through their contacts with the PsbO, PsbQ' and Psb31 proteins. Recently, we showed a close association of the Psb31 protein with the PsbH protein and/or Cyt *b*559α in the diatom PSII core complex by cross-linking experiments using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) (31). Based on these facts and the crystal structure of cyanobacterial PSII currently available (11, 40-42), PsbO was placed on the lumenal surface with a broad contact with CP47, CP43, D1 and D2 subunits in Fig. 1C. Psb31 is also situated in close to the D1/D2 proteins through its direct association with PsbH and/or Cyt *b*559α. PsbQ' is tentatively located in the CP47 side, because there is no space in the CP43 side due to overlapping of the structure of PsbO with CP43 (11, 40-42). PsbV and PsbU have contacts with all of the other extrinsic proteins and partially with some of the PSII intrinsic proteins. Although the detailed organization of these extrinsic proteins in diatom PSII has to wait for a high resolution

crystal structure to be determined, before such a structure is available, the model proposed in this study will provide useful information for the arrangement of the relevant subunits in diatom PSII.

Function of the five extrinsic proteins in diatom PSII - One significant feature of the diatom PSII revealed by this study is that diatom PSII reconstituted with the novel Psb31 protein can evolve oxygen even in the absence of the PsbO protein (Tables 5 and 6). Among the extrinsic proteins in PSIIs from a wide variety of organisms, the PsbO protein plays an important role in maintaining the stability and activity of the Mn_4Ca-cluster, and has been considered to be absolutely essential for oxygen evolution (1-17, 29, 30, 37-39). This study is the first report demonstrating that PSII lacking PsbO can evolve oxygen in the absence of Cl^- and Ca^{2+} ions. As shown in Tables 5 and 6, PSII reconstituted with Psb31 alone showed an oxygen-evolving activity of 128 μmol O_2/mg Chl/h in the absence of Cl^- and Ca^{2+} ions, compared with 173 μmol O_2/mg Chl/h in PSII reconstituted with PsbO alone. Furthermore, PSII reconstituted with Psb31 in combination with PsbQ', PsbV and PsbU showed the activity of 343-470 μmol O_2/mg Chl/h in the absence of PsbO (Table 6). These indicate that the novel Psb31 extrinsic protein partially serves as a substitute for the PsbO protein in supporting oxygen evolution. The significant difference between PSIIs reconstituted with Psb31 alone and with PsbO alone is that the oxygen-evolving activity of the former is scarcely stimulated by Cl^- and Ca^{2+} ions but that of the latter is largely stimulated by these ions (Tables 5 and 6). This may suggest that the binding of Psb31 has shielded the access of Cl^- and Ca^{2+} to their functional sites close to the Mn_4Ca-cluster, whereas binding of PsbO did not. In any event, it is an interesting subject to be elucidated as to why the activity of PSII reconstituted with PsbO was largely stimulated by Cl^- and Ca^{2+} ions but that of PSII reconstituted with Psb31 was not.

In PSII reconstituted with PsbO, rebinding of PsbV, or PsbV and PsbU largely activated the oxygen evolution in the absence of Ca^{2+} ion, or both Cl^- and Ca^{2+} ions, respectively (Table 6), which is similar to that reported in the cyanobacterial and red algal PSIIs (1, 2, 5, 12). These have been interpreted to indicate that PsbV and PsbU function to optimize the availability of Cl^- and Ca^{2+} cofactors for water oxidation, with PsbV mainly functioning to optimize the availability of Ca^{2+} and PsbU mainly functioning to optimize Cl^-, respectively. The oxygen evolution in the absence of Ca^{2+} ion or in the absence of both Cl^- and Ca^{2+} ions was also activated in PSII reconstituted with Psb31 by rebinding of PsbV, or PsbV and PsbU, respectively (Table 6). These indicate that the function of the PsbV and PsbU proteins in activating oxygen evolution in the absence of Cl^- and Ca^{2+} ions is retained in PSII reconstituted with Psb31, similar to those of PSII reconstituted with PsbO.

The restoration of oxygen evolution in PSII reconstituted with all of the five extrinsic proteins was only 20-23% of the original activity when release-reconstitution experiments were carried out under normal condition, whereas the restoration increased to 46-50% when the experiments were performed under Mn-photoactivation condition (Table 1). This suggests that Mn ions are partially liberated by the 4 M urea plus 0.2 M NaCl treatment, or during incubation following the treatment, as two out of the four Mn atoms per PSII have been reported to be gradually liberated after 24-48 h when the extrinsic proteins-depleted PSII was incubated at 0ºC in the dark (43, 44). The Mn-liberation was shown to be suppressed by rebinding of PsbO (43, 44). Our preliminary experiments on inactivation of oxygen evolution during incubation on ice in the dark, however, showed that all of the extrinsic proteins in addition to PsbO were required for stabilization of the oxygen-evolving activity of the diatom PSII, suggesting that the Psb31 protein may contribute to stabilize the manganese cluster together with PsbO.

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FOOTNOTES

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The abbreviations used are: CBB, Coomassie Brilliant Blue R-250; Chl, chlorophyll; Cyt *b*559α, α subunit of cytochrome *b*559; DCIP, 2,6-dichloroindophenol; EDTA, ethylenediaminetetraacetic acid; FCP, fucoxanthin chlorophyll *a*/*c*-binding proteins; MES, 2-morpholinoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBQ, phenyl-*p*-benzoquinone; PMSF, phenylmethyl sulfonyl fluoride; PSII, Photosystem II; RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenases

FIGURE LEGENDS

FIGURE 1. **Schematic models of PSIIs from cyanobacteria (A), red algae (B), diatom (C), green alga and *euglena* (D), and higher plants (E), showing the species of various extrinsic proteins and their direct and indirect associations with PSII at the lumenal side based on various reconstitution and cross-reconstitution studies.** See text for details.

FIGURE 2. **Purification of the five extrinsic proteins from crude PSII of a marine centric diatom, *Chaetoceros gracilis*.** Lane 1, crude PSII; lane 2, five extrinsic proteins extracted from the crude PSII by alkaline Tris-treatment; lane 3, purified PsbO; lane 4, purified PsbQ'; lane 5, purified PsbV; lane 6, purified Psb31; lane 7, purified PsbU. The gel was stained with CBB, and each polypeptide was identified by immunological and/or N-terminal sequence analyses according to the methods described previously (29, 30).

FIGURE 3. **Dissociation of extrinsic proteins from purified PSII (lane 1) of a marine centric diatom, *Chaetoceros gracilis*, by treatment with 1 M NaCl (lane 2), 1 M $MgCl_2$ (lane 3), 1 M Tris-HCl (pH 8.5) (lane 4), 2.6 M urea/0.2 M NaCl (lane 5), or 4 M urea/0.2 M NaCl (lane 6).** "S" and "P" stand for "supernatant" and "pellet", respectively.

FIGURE 4. **Reconstitution of urea/NaCl-treated PSII with each or all of the five extrinsic proteins.** Lane 1, purified PSII from a marine centric diatom, *Chaetoceros gracilis*; lane 2, 4 M urea/0.2 M NaCl-treated PSII; lanes 3-8, the urea/NaCl-treated PSII reconstituted with PsbO (lane 3), PsbQ' (lane 4), PsbV (lane 5), Psb31 (lane 6), PsbU (lane7) and all of the five extrinsic proteins (lane 8).

TABLE 1

Oxygen evolution of the urea/NaCl-treated PSII reconstituted with all of the five extrinsic proteins under normal condition and the Mn-photoactivation condition

	Oxygen evolution (µmol O_2/mg Chl/h) [a]		
	- ions	+ 10 mM NaCl	+ 5 mM $CaCl_2$
untreated PSII	2143 ± 123 (100)	2240 ± 117 (100)	2240 ± 140 (100)
urea/NaCl-treated PSII	0 (0)	90 ± 9 (4)	134 ± 19 (6)
+ all of the extrinsic proteins (normal condition)[b]	450 ± 43 (21)	448 ± 43 (20)	515 ± 40 (23)
+ all of the extrinsic proteins (Mn-photoactivation condition)[c]	988 ± 74 (46)	1113 ± 66 (50)	1130 ± 90 (50)

[a] Values are shown as mean ± S.D., with n = 3 (3 independent experiments)

[b] Reconstitution experiments were carried out in a medium containing 0.4 M sucrose and 40 mM MES-NaOH (pH 6.5) (buffer B) on ice in the dark.

[c] Reconstitution experiments were carried out in buffer B containing 10 µM DCIP, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 10 mM $CaCl_2$ on ice at room light (7-8 µmol photons/m^2/s).

TABLE 2

Relative binding of PsbV reconstituted to urea/NaCl-treated PSII in various combinations with the other extrinsic proteins

	Amount of PsbV rebound (%) [a]
PsbV only	14 ± 2
PsbV + PsbO	42 ± 8
PsbV + PsbQ’	56 ± 11
PsbV + Psb31	54 ± 10
PsbV + PsbO + PsbQ’	65 ± 10
PsbV + PsbQ’ + Psb31	72 ± 11
PsbV + PsbO + Psb31	61 ± 12
PsbV + PsbO + PsbQ’ + Psb31	84 ± 15
PsbV + PsbO + PsbQ’ + Psb31 + PsbU	101 ± 3

[a] Values are shown as mean ± S.D., with $n = 6$ (2 times of Western analysis for each of 3 independent samples)

TABLE 3

Relative binding of PsbU reconstituted to urea/NaCl-treated PSII in various combinations with the other extrinsic proteins

	Amount of PsbU rebound (%) [a]
PsbU only	5 ± 3
PsbU + PsbO	34 ± 8
PsbU + PsbQ’	30 ± 8
PsbU + Psb31	34 ± 8
PsbU + PsbO + PsbQ’	41 ± 9
PsbU + PsbQ’ + Psb31	54 ± 10
PsbU + PsbO + Psb31	66 ± 8
PsbU + PsbO + PsbQ’ + Psb31	78 ± 11
PsbU + PsbO + PsbQ’ + Psb31 + PsbV	103 ± 4

[a] Values are shown as mean ± S.D., with n = 6 (2 times of Western analysis for each of 3 independent samples)

TABLE 4

Relative binding of PsbV and PsbU reconstituted to urea/NaCl-treated PSII in various combinations with the other extrinsic proteins

	Amount of PsbV rebound (%) [a]	Amount of PsbU rebound (%)[a]
PsbV + PsbU	56 ± 11	23 ± 6
PsbV + PsbU + PsbO	68 ± 13	55 ± 8
PsbV + PsbU + PsbQ’	76 ± 14	52 ± 8
PsbV + PsbU + Psb31	72 ± 15	61 ± 8
PsbV + PsbU + PsbO + PsbQ’	80 ± 15	73 ± 9
PsbV + PsbU + PsbQ’ + Psb31	87 ± 15	77 ± 8
PsbV + PsbU + PsbO + Psb31	82 ± 16	84 ± 12
PsbV + PsbU + PsbO + PsbQ’ + Psb31	100 ± 3	102 ± 3

[a] Values are shown as mean ± S.D., with n = 6 (2 times of Western analysis for each of 3 independent samples)

TABLE 5

Oxygen evolution of the urea/NaCl-treated PSII reconstituted with the extrinsic proteins in various combinations under the Mn-photoactivation condition

	Oxygen evolution (μmol O_2/mg Chl/h) [a]		
	- ions	+ 10 mM NaCl	+ 5 mM $CaCl_2$
PSII	2143 ± 123 (100)	2240 ± 117 (100)	2240 ± 140 (100)
urea/NaCl-treated PSII	0 (0)	90 ± 9 (4)	134 ± 19 (6)
+ PsbO	173 ± 17 (8)	537 ± 35 (24)	985 ± 62 (44)
+ PsbQ'	0 (0)	112 ± 13 (5)	134 ± 18 (6)
+ Psb31	128 ± 12 (6)	150 ± 15 (7)	156 ± 19 (7)
+ PsbO + PsbQ'	186 ± 13 (9)	542 ± 38 (24)	1044 ± 78 (47)
+ PsbO + Psb31	169 ± 17 (8)	588 ± 41 (26)	979 ± 77 (44)
+ PsbO + PsbQ' + Psb31	173 ± 24 (8)	603 ± 40 (27)	1070 ± 61 (48)
+ PsbO + PsbQ' + Psb31 + PsbV	283 ± 27 (13)	808 ± 42 (36)	1012 ± 72 (45)
+ PsbO + PsbQ' + PsbV + Psb31 + PsbU	988 ± 74 (46)	1113 ± 66 (50)	1130 ± 90 (50)

[a] Values are shown as mean ± S.D., with $n = 3$ (3 independent experiments)

TABLE 6

Oxygen evolution of the urea/NaCl-treated PSII reconstituted with the other extrinsic proteins in various combinations with the Psb31 or PsbO protein under the Mn-photoactivation condition

	Oxygen evolution (μmol O_2/mg Chl/h) [a]		
	- ions	+ 10 mM NaCl	+ 5 mM $CaCl_2$
untreated PSII	2143 ± 123 (100)	2240 ± 117 (100)	2240 ± 140 (100)
urea/NaCl-treated PSII	0 (0)	90 ± 9 (4)	134 ± 19 (6)
+ Psb31	128 ± 12 (6)	150 ± 15 (7)	156 ± 19 (7)
+ Psb31 + PsbQ’	151 ± 19 (7)	186 ± 16 (8)	183 ± 21 (8)
+ Psb31 + PsbQ’ + PsbV	159 ± 14 (7)	292 ± 23 (13)	320 ± 26 (14)
+ Psb31+ PsbQ’ + PsbV + PsbU	343 ± 22 (16)	452 ± 31 (20)	470 ± 31 (21)
+ PsbO	173 ± 17 (8)	537 ± 35 (24)	985 ± 62 (44)
+ PsbO + PsbQ’	186 ± 13 (9)	542 ± 38 (24)	1044 ± 78 (47)
+ PsbO + PsbQ’ + PsbV	257 ± 20 (12)	672 ± 36 (30)	1075 ± 80 (48)
+ PsbO + PsbQ’ + PsbV + PsbU	711 ± 56 (33)	784 ± 62 (35)	1081 ± 86 (48)

[a] Values are shown as mean ± S.D., with $n = 3$ (3 independent experiments)

Fig. 1 Nagao et al.

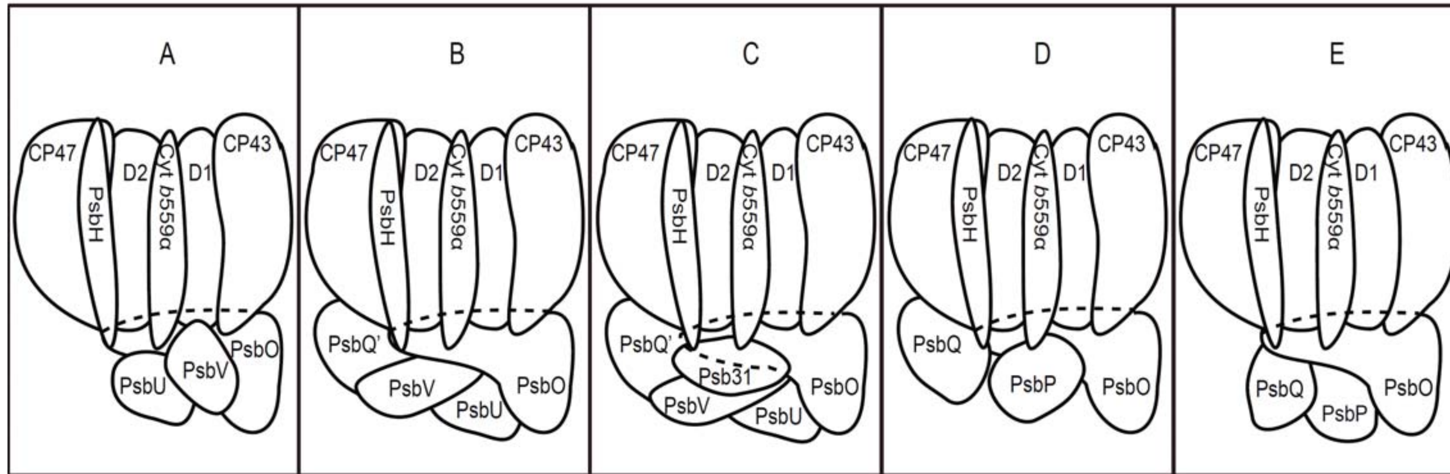

Fig. 2 Nagao et al.

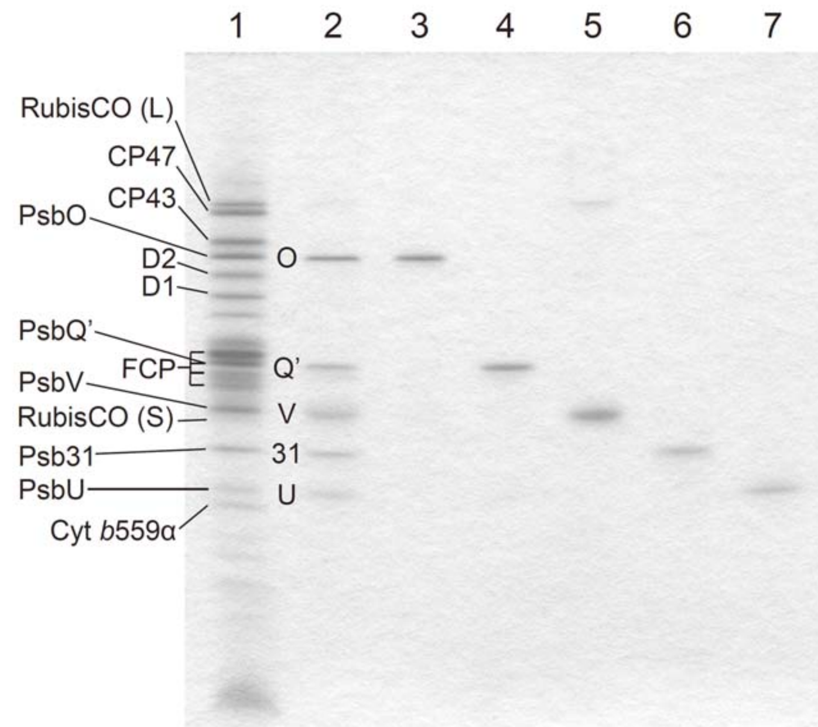

Fig. 3 Nagao et al.

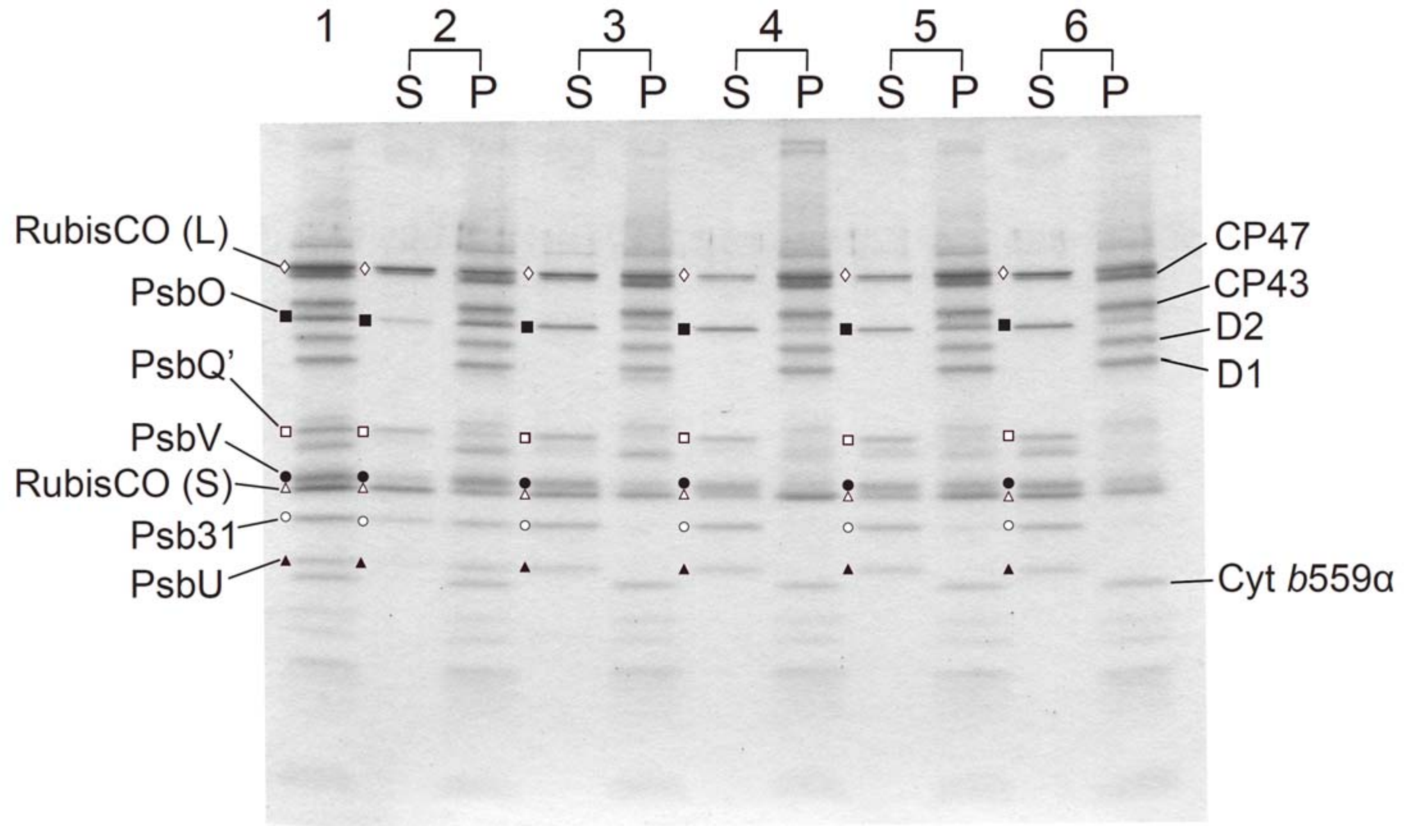

Fig. 4 Nagao et al.