Three-dimensional Electron Cryo-microscopy Study of the Extrinsic Domains of the Oxygen-evolving Complex of Spinach

ASSIGNMENT OF THE PsbO PROTEIN*

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Three independent three-dimensional reconstructions of the spinach photosystem II-light-harvesting complex supercomplex were derived from single particle analyses of non-stained, vitrified samples imaged by electron microscopy. Each reconstruction was found to differ significantly in the composition of the luminal oxygen-evolving complex extrinsic proteins. From difference mapping, aided by electron microscopy of negatively stained selectively washed samples, regions of density were assigned to the PsbO and PsbBP/PsbQ proteins. Interpretation of the density assigned to the PsbO protein was explored using computer-aided structural predictions. PsbO is calculated to be mainly a β-protein (38% β) composed of two domains within an overall elongated shape (Pazos, F., Heredia, P., Valencia, A., and De Las Rivas, J. (2001) Proteins Struct. Funct. Genet. 45, 372–381). The positioning and fitting of the proposed structural model for the PsbO protein within the three-dimensional map indicated that there is a single copy per reaction center. Moreover, the structural model derived for PsbO, together with difference mapping, indicates that this protein stretches across the surface of the reaction center with its N- and C-terminal domains located toward the CP47 and CP43 side, respectively. This structural assignment is discussed in terms of the recent x-ray-derived cyanobacterial model of PSII (Zouni, A., Witt, H.-T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) Nature 409, 739–743).

Plants, algae, and cyanobacteria are able to use the energy of visible light to break the O–H bond of water and produce molecular oxygen. Photosystem II (PSII),¹ a multi-subunit transmembrane protein complex (1), carries out this difficult and thermodynamically demanding reaction. The water oxidation activity of PSII is driven by a series of electron transfer reactions. Light-induced primary charge separation gives rise to the radical pair P680++/Pheo− where P680 is a high potential form of chlorophyll a (1 V or more) and Pheo is a pheophytin a molecule. Electron flow proceeds from Pheo− to the plastoquinone molecules QA and QB. When QA is doubly reduced, it is protonated and replaced by an oxidized plastoquinone (PQ) molecule from the PQ pool in the membrane. On the electron donor side, P680++ is reduced by a tetramanganese cluster (Mn)₄ via a redox-active tyrosine Y₉. These redox-active cofactors are bound to the D1 and D2 reaction center proteins and are arranged in such a way that electron flow occurs across the membrane with the water oxidation site located on the luminal/periplasmic surface and PQ reduction close to the stromal/cyttoplasmic surface (2).

The (Mn)₄ cluster is the catalytic site for water oxidation and is ligated to the D1 protein in the region of Y₉ (D1 Tyr-161) (3). This inorganic group is stabilized by an extrinsic 33-kDa protein (PsbO) encoded by the psbO gene. Also associated with the oxygen-evolving complex (OEC) are at least two other extrinsic proteins, the 23-kDa (PsbP) and 17-kDa (PsbQ) proteins in plants and green algae and the 15-kDa (PsbV) and 11-kDa (PsbU) proteins in cyanobacteria (4). Together the OEC extrinsic proteins provide an optimal ionic (Cl⁻ and Ca²⁺) environment for the water oxidation reaction (3), although there is a lack of clear knowledge about the specific function and activity of these proteins during the process of water oxidation and oxygen evolution. In the case of higher plants, little is known about the structure of the OEC proteins (5) or about their specific location and arrangement in relation to the intrinsic subunits of PSII. In the case of the PsbO protein, there have been assignments of its secondary structure based on spectroscopic studies (6). Fourier transform infrared spectroscopy has indicated that the 33-kDa protein is composed predominantly of β-structure (7–10), a conclusion reinforced by circular dichroism (CD) measurements in the ultraviolet region (11, 12). Structural predictions also suggest that this is a β-protein consisting of nine or more β-strands with a high proportion of loops and turns (6, 13, 14). These predictions have recently been used to assign a region of the x-ray-derived map of the PSII core of Synechococcus elongatus to a portion of the PsbO protein (15).

Less is known about the structure of the PsbP and PsbQ extrinsic OEC proteins present in higher plants and algae (5). This is also true for the PsbU extrinsic protein of cyanobacteria, but a high resolution three-dimensional structure of PsbV (Cyt-549) of Synechocystis has recently been obtained (16), and its assignment within the x-ray map of PSII of S. elongatus is unequivocal (15).

Studies of PSII using electron microscopy (EM) and single particle analyses have provided low resolution three-dimensional structural information for the OEC proteins of spinach (17) and Chlamydomonas reinhardtii (18). In the case of the

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1 The abbreviations used are: PSII, photosystem II; LHCII, light-harvesting complex; OEC, oxygen-evolving complex; EM, electron microscopy.
former, the three-dimensional structure was calculated to 24-Å resolution (using vitrified samples), and the extrinsic OEC proteins were observed as luminal density extending from the membrane surface. We have refined the density map of the spinach PSII supercomplex to 17 Å, and in this study, the data are presented with special focus on the region assigned to the PsbO protein on the luminal side of PSII. The results obtained are discussed in terms of structural predictions for the PsbO protein based on computational methodologies (14) and the x-ray structure of cyanobacterial PSII published recently in Ref. 16.

MATERIALS AND METHODS

Image Processing—An improved three-dimensional model of the spinach PSII supercomplex has been derived and compared with that reported in Nield et al. (17). To summarize, a vitrified sample was imaged using a Philips CM200 FEG EM, and the technique of single particle analysis was implemented via the Image-V software environment (19). We identified, from over 100 micrographs, 10,150 supercomplex particles that differed only in their luminal extrinsic protein mass. Refinement of this data set by classification (20) identified 6,100 particles in the largest subpopulation followed by two further subpopulations of 2,900 and 1,150 particles. Each subpopulation was treated de novo, resulting in three-dimensional maps calculated by angular reconstruction (21) and subsequent exact back projection (20). For the negatively stained EM study, the procedures used were as detailed previously (23, 24) where a Philips CM100 EM was used at room temperature. Here, LHCII-PSII supercomplexes isolated from PSI-, enriched membranes were either left untreated or washed with 150 mM NaCl to remove the PsbP and PsbQ proteins or with 1 μTris (pH 8.0) to remove PsbO, PsbP, and PsbQ, in each case prior to the β-n-dodecyl maltoside solubilization step (17). Five micrographs from each preparation were chosen, resulting in data sets of ~1,000 particles for NaCl-, Tris-, or non-washed preparations, respectively. The application of classification and two-dimensionally averaging procedures (20) allowed characteristic views to be identified and refined. Structural coordinates derived from the 3.8-Å x-ray model of PSI for S. elongatus (15), designated under the Research Collaboratory for Structural Bioinformatics (RCSB) (www.rcsb.org/) Protein Data Bank code 1FE1, were modeled into the three-dimensional reconstructions using the “O” software package (22).

Protein Structural Predictions and Threading—A structural model of PsbO has been obtained (14). This model was constructed based on remote homology using a protocol that follows three progressive criteria for a correct assignment: 1) reliability and consistency of fold recognitions obtained using seven different threading methods (TOPITS, GONPM, H3P2, 3-PSSM, HFR, THREADER2, and HMM); 2) quantitative evaluation of the fitting of the different Protein Data Bank templates selected to the internal properties of the target protein, PsbO (the properties studied were: clustering of apolar residues, clustering of conserved residues, and conservation of correlated residues and of tree-determinant amino acids); and 3) consistency or agreement of the predicted model with experimental data known about the structure of the protein. Each threading method used a specific approach to identify Protein Data Bank candidates that showed significant fold homology with the target protein. The use of a combination of methods improves the reliability of the threading predictions. The best Protein Data Bank candidates for PsbO were selected and used as templates to construct the three-dimensional models. Models were built using SWISS-MODEL-ELLER and internally evaluated with the automatic tool WHATIF-Check. Threading models were then evaluated according to the fitting of sequence-extracted features derived from the study of the PsbO protein family. Finally, experimental structural data that are available for PsbO were used to recheck the model. The basic principles of each threading method used and full details of the PsbO fold recognition and model construction are detailed in Pazos et al. (14).

RESULTS

17 Å Three-dimensional Map of the LHCII-PSII Supercomplex Isolated from Spinach—A surface-rendered oblique view of the structure of the LHCII-PSII supercomplex of spinach, obtained by single particle analysis of images recorded by electron microscopy at cryogenic temperature, is shown in Fig. 1. The resolution of this structure is estimated to be 17 Å and is therefore an improvement on that of Nield et al. (17). This improved resolution is in part due to extending the data set from 5,300 to 10,150 particles and to further refinement based on investigating the presence or absence of extrinsic lumina bound proteins. Classification identified 6,100 particles with a full complement of extrinsic proteins. We have previously attributed the centrally located luminal protrusion (A/A’) to the 33-kDa (PsbO) subunit (23), whereas B/B’ was suggested to accommodate both the 23-kDa PsbP protein and the 17-kDa PsbQ protein (17, 18, 24). These assignments have been confirmed further by conducting electron microscopy on negatively stained LHCII-PSII supercomplexes from spinach that had been either washed with 150 mM NaCl to remove the 23-kDa PsbP and 17-kDa PsbQ or with 1 μTris (pH 8.0) to remove all three OEC extrinsic proteins. This analysis focused on side views, and the averaged images are shown in Fig. 2. As found previously in the absence of the three OEC proteins, the luminal surface appears relatively featureless at the resolution of the analysis (Fig. 2C). However, when the 33-kDa PsbO protein is present, there is a centrally located protrusion (Fig. 2B) that was assigned previously to the overlapping side projection of two copies of this protein (18, 24). Only when all three OEC proteins are present were the densities directly adjacent to the centrally located protrusion observed (Fig. 2A). Therefore these studies support the assignments made previously for these structural features in the three-dimensional map (17, 23, 24). Two copies of the density attributed to the OEC proteins are present because the LHC-PSII supercomplex is a dimer having two reaction centers per complex (23). The three-dimensional reconstruction of the supercomplex therefore exploited this symmetry. Note that after Tris (pH 8.0) washing, the supercomplex no longer aggregates as a dimer of dimers (Fig. 2C).

The overall dimensions of the intact supercomplex are 330 Å (length) × 165 Å (width) × 110 Å (height), and the new three-dimensional cryo-EM map is suitable for a closer examination of the density assigned to the OEC proteins. Indeed, Fig. 3 gives various close-up views of the protrusions attributed to the OEC extrinsic proteins. The tetrameric organization of these protrusions at the surface of the dimeric supercomplex is reminiscent of that revealed earlier by freeze-etch studies (25, 26). The densities assigned to PsbP and PsbQ extend about 50 Å from the luminal surface of the supercomplex, whereas the density attributed to the PsbO protein is about 40 Å from the surface. Fig. 3, A and F, indicates that the OEC proteins of one monomeric PSII complex do not make direct contact with the complementary OEC proteins of the other monomer within the dimeric supercomplex.

Fig. 4 shows four projections of 2-Å-thick cross-sections of the
OEC proteins at various distances from the lumenal surface. The cross-sections have been taken as indicated in Fig. 3 and clearly show the tetrameric organization of the proteins and the increase in their overall density on approaching the membrane surface. The cross-section closest to the lumenal surface (cross-section 1) shows the density distribution for the surface of the underlying core dimer to which the OEC proteins bind, which is almost certainly composed of the lumenal loops of the transmembrane segments of the intrinsic proteins, particularly the large loops joining helices 5 and 6 of CP43 and CP47 (4, 27).

In cross-section 2, a density feature is clearly seen (Fig. 4, arrow) that is elongated (50 Å or more long), stretching across a significant portion of the underlying monomer. This density is attributed to the PsbO protein (see below).

**PSII Supercomplexes with and without the OEC Proteins**—Bearing in mind earlier EM analyses of samples with different complements of extrinsic OEC proteins (17, 18, 23, 24) and the new data shown in Fig. 2, it was possible to extract from the total data set of 10,150 particles observed by cryo-EM ~2,900 particles having no OEC protrusions and 1,150 particles that had lost the density attributed to the PsbP and PsbQ proteins. Oblique views of the PsbP/PsbQ (Fig. 5B) and PsbO/PsbP/PsbQ-depleted (Fig. 5C) supercomplexes allow comparison of their three-dimensional structures with the intact supercomplex (Fig. 5A). Although the resolutions of the three-dimensional structure of the OEC-depleted (21 Å) and the PsbP/PsbQ-depleted (28 Å) supercomplex are poorer than the intact supercomplex (17 Å), they do provide a framework for interpreting the densities on the lumenal surface in terms of the OEC proteins. Indeed, the difference maps presented in Fig. 6 provide information about the dimensions and angular orientations of the extrinsic proteins and emphasizes that they are located about 12 Å above the lumenal surface with the PsbP/PsbQ and the PsbO volumes extending a further 42 and 32 Å, respectively. The 12-Å region immediately above the lumenal surface presumably accommodates the loops joining the underlying transmembrane helices. When the PsbP and PsbQ proteins are not present, it can be seen that the density assigned to the PsbO stretches across the stromal surface of the complex (Fig. 6B, colored in cyan).

**Structural Model of PsbO**—Given the volume available for the OEC proteins, we have suggested previously that the protrusions were likely to accommodate one copy each of PsbO, PsbP, and PsbQ proteins per PSII monomer within the LHClII-PSII supercomplex (17, 18). Structure and fold predictions for these proteins would provide a means to check this.
conclusion and further open up discussion of their functional roles. However, only in the case of the PsbO protein have such predictions been undertaken. The most recent and thorough model has been calculated by Pazos et al. (14). This structural model of PsbO is based on fold recognition and molecular modeling. It predicts PsbO as an all-\beta-protein with two homologous \β-domains of \( \sim 120 \) amino acids linked by a centrally located, flexible Pro-Gly-Gly motif. The deduced overall shape for PsbO indicates a cylindrical-like, elongated molecule having a length of \( \sim 80 \) Å and a diameter of 20–30 Å. The N-terminal domain is predicted to include eight or nine \β-strands, and the C-terminal domain is predicted to include six or seven \β-strands. The two domains are about 32–40 Å long and form a sandwich-type structure with Greek-key topology linked by a single loop. This arrangement could explain the observations that have indicated PsbO to be a highly flexible protein (6). For example, it undergoes conformational changes upon binding to PSII (28) and does not have a well defined structure when observed in solution (29, 30). However, dynamic light-scattering and ultracentrifugation studies on the PsbO protein (31) have indicated that this protein has a prolate, ellipsoid shape.

The C-terminal domain has two long loops, Gly-152–Gly-163 and Gly-177–Gln-190 (spinach sequence), which were not included in the model of Pazos et al. (14) given that they are very distinct for PsbO. The former includes a highly conserved region (between Pro-148 and Pro-174) containing several charged residues (Asp-157, Lys-159, Arg-161) that have been related to the specific interaction and binding of PsbO to PSII and probably to the stabilization of the manganese cluster (6, 32). The conserved Pro-148–Pro-174 region also includes a motif Glu-X-Asp-Glu-Glu-Asp, which is very similar to the calcium-binding motif identified in PROSITE as PS00330.

Matching the PsbO Model with Attributed Density—In Fig. 7, we show a fit of the computer-derived remote model of the PsbO protein of Pazos et al. (14) into the density that we attribute to this OEC protein within the three-dimensional cryo-EM map. The fitting has been conducted based on the overall shape and size of the model with constraints imposed by the volume available. We have also incorporated the three-dimensional structural model of the PSII core dimer complex of \textit{S. elongatus} derived from x-ray crystallography by Zouni et al. (15) including the proposed backbone for the PsbV (Fig. 7B, cV) and a
portion of the PsbO (Fig. 7B, cO) proteins. The core dimer is positioned centrally within the LHCl-II-PSII supercomplex as described previously (17, 18, 33). In so doing, we are able to relate the extrinsic mass with the approximate positions of the underlying transmembrane helices of the various PSII subunits (Fig. 7A) and in particular, identify the location of the manganese cluster within the cryo-EM map.

**Assignment of PsbO Protein**

Using cryo-EM and difference mapping, we have attempted to identify the positioning of the 33-kDa protein within the three-dimensional structure of the LHCl-II-PSII supercomplex of spinach. The interpretation of the density attributed to the PsbO has been aided by comparison with a recently derived structural model of this protein (14). Our analysis of the EM data is consistent with the PsbO protein having an elongated cylindrical shape and suggest that it lies along the lumenal surface of the PSII core. As discussed in earlier papers (17, 18), it is possible to incorporate the intrinsic components of the central core dimer of *S. elongatus* into the EM structure of the LHCl-II-PSII supercomplex. The earlier modeling relied on the data obtained by electron crystallography (34–36), but in Fig. 7, we have used the recently elucidated x-ray structure of PSII from *S. elongatus* (15). In so doing, we conclude that the PsbO protein is located above the luminal ends of the helices of the D1 and D2 reaction center proteins and their corresponding cd surface helices. Moreover, because of its elongated shape, the PsbO protein also lies above helices 5 and 6 of CP47 and to one side of the transmembrane helices of CP43.

The x-ray structure of the PSII complex of *S. elongatus* also identified the positioning of the (Mn)4 cluster. According to the EM map, this metal cluster is located about 8 Å below the PsbO protein. Based on this, it would seem unlikely that this OEC protein provides ligands for manganese, a conclusion supported by the fact that PsbO deletion mutants of Synechocystis 6803 are able to evolve oxygen and grow phototrophically (37, 38). However, as mentioned above, there is evidence that the C-terminal domain of the PsbO protein is the main domain involved in interacting with and stabilizing the (Mn), cluster, and for this reason, the N-terminal region is assumed to be positioned toward the CP47 side of the PSII complex. As can be seen in Fig. 7A, the proposed N-terminal region of the PsbO protein is located over the two transmembrane domains assigned to helices 5 and 6 of CP47 (36). These two helices are joined by a loop containing ~200 amino acids (39). Indeed, there are many studies indicating that the PsbO protein binds, in part, to the luminal exposed regions of CP47, particularly the large loop joining transmembrane helices 5 and 6 (6). Several of these studies suggest that it is the N-terminal end of the PsbO that interacts with the CP47 protein. For example, Odom and Bricker (40) concluded from cross-linking studies using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide that the N-terminal domain Glu–Lys–76 of the PsbO protein is located close to the Glu–364–Asp–440 region in the large lumenal loop of CP47.

Although the elucidation of the structure of the cyanobacterial PSII by x-ray diffraction has been a major step forward, its poor resolution (3.8 Å) has not allowed a full structural description of the OEC proteins to be made (15). For example, only half of the Ca backbone of PsbO is identified, and no information has emerged about the structure and positioning of the PsbU protein. The most reliable outcome of the x-ray analyses was the identification of the density of the PsbV protein (Fig. 7B, cV) and the iron atom of its heme. In higher plants, such as spinach, the PsbV and the PsbU protein have been functionally replaced by the PsbP and PsbQ proteins. However, according to our analyses, the PsbP/PsbQ proteins, unlike the PsbV, are located above the luminal ends of the CP43 transmembrane helices (Fig. 7B, purple arrow on the left). For this reason, they are immediately adjacent to the PsbO protein, which is consistent with studies that have shown that the binding of the PsbP to PSII requires the presence of the PsbO and that PsbP is required for the binding of PsbQ (41). In contrast, the PsbV can bind to PSII in the absence of the PsbO protein (42), which is
is possible that the conformation of the PsbO protein changes in the absence of the PsbP/PsbQ proteins. This is unlikely, however, since the density assigned to the PsbO protein and the calculated model seems to adequately fit into the 17-Å three-dimensional cryo-map of the supercomplex having its full complement of OEC proteins.

4) It is not inconceivable that the PsbO protein could have different conformational states as part of its functional activity. For example, it has recently been shown to bind GTP (43), suggesting that it may have additional functions other than stabilizing the (Mn)4 cluster.

5) Given that the other OEC proteins of higher plants and cyanobacteria are distinctly different, there is the possibility that the conformational state of the PsbO proteins are different for the two systems.

Whatever the explanation, the work presented here does highlight the need to recognize that the PsbO is an unusually flexible protein that may well undergo large conformational changes even when bound to the luminal surface of PSII. It could be this property that gives rise to the difference between the conclusions deduced from the EM work described here and the x-ray analyses reported recently (15). Moreover, changes in the conformation of the PsbO protein could explain the biphasic kinetics for the binding of PsbO to PSII that, together with other studies, led to the idea of there being two copies of this protein per reaction center (6). Although compelling evidence has been presented to suggest that there are two copies of the PsbO protein per PSII (reviewed in Ref. 6), in our structural studies, we have found insufficient density to account for this proposed stoichiometry. In fact, since it is generally agreed that the stoichiometry ratio of the three extrinsic proteins (PsbO, PsbP, and PsbQ) is 1:1:1 (6, 41), then the expected luminal density would be far greater than observed by us. However, it could be argued that additional copies of the extrinsic proteins were lost during the isolation of the LHClI-PSII supercomplex. However, this seems unlikely since the luminal densities assigned to the OEC proteins are very similar in organization and size to those found by Seibert et al. (25) using freeze-etch techniques and intact membranes. Moreover, a stoichiometry of one copy of each OEC protein has been implied by the x-ray studies of Zouni et al. (15) using PSII from S. elongatus.

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