Inorganic Cofactor Stabilization and Retention: The Unique Functions of the Two PsbO Subunits of Eukaryotic Photosystem II[†]

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ABSTRACT: Eukaryotic PsbO, the photosystem II (PSII) manganese-stabilizing protein, has two N-terminal sequences that are required for binding of two copies of the protein to PSII [Popelkova, H., et al. (2002) *Biochemistry 41*, 10038−10045; Popelkova, H., et al. (2003) *Biochemistry 42*, 6193−6200]. In the work reported here, a set of selected N-terminal truncation mutants of PsbO that affect subunit binding to PSII were used to determine the effects of PsbO stoichiometry on the Mn, Ca^{2+}, and Cl^- cofactors and to characterize the roles of each of the PsbO subunits in PSII function. Results of the experiments with the PsbO-depleted PSII membranes reconstituted with the PsbO deletion mutants showed that the presence of PsbO does not affect Ca^{2+} retention by PSII in steady-state assays of activity, nor is it required for Ca^{2+} to protect the Mn cluster against reductive inhibition in darkness. In contrast to the results with Ca^{2+}, PsbO increases the affinity of Cl^- for the active site of the O_2-evolving complex (OEC) as expected. These results together with other data on activity retention suggest that PsbO can stabilize the Mn cluster by facilitating retention of Cl^- in the OEC. The data presented here indicate that each of two copies of PsbO has a distinctive function in PSII. Binding of the first PsbO subunit fully stabilizes the Mn cluster and enhances Cl^- retention, while binding of the second subunit optimizes Cl^- retention, which in turn maximizes O_2 evolution activity. Nonspecific binding of some PsbO truncation mutants to PSII has no functional significance.

The photosystem II (PSII)[1] O_2-evolving complex (OEC), the catalyst of the H_2O oxidation reaction, consists of three inorganic cofactors (one Ca^{2+}, one Cl^-, and four Mn atoms) that are shielded from the thylakoid lumen by three extrinsic polypeptides attached to the intrinsic subunits of PSII. The largest extrinsic protein, called PsbO (33 kDa) or manganese-stabilizing protein, is present in all O_2-evolving photosynthetic organisms. The two smaller extrinsic polypeptides in higher plants and algae, PsbP (23 kDa) and PsbQ (17 kDa), are replaced by PsbV (cyt 550) and PsbU (12 kDa) in cyanobacteria (*1*). Cyanobacterial homologues of PsbP and PsbQ have also been discovered in *Synechocystis* sp. PCC 6803 (*2*); their function is still under investigation. Binding of the PsbP and PsbQ polypeptides in eukaryotic PSII facilitates retention of Ca^{2+} and Cl^- by the OEC (*3*−*5*). Removal of these polypeptides from PSII inhibits O_2 evolution activity; addition of Ca^{2+} and Cl^- restores up to 75% of the intact PSII activity (*3*).

In contrast, the largest extrinsic polypeptide, PsbO, is essential for both O_2 evolution activity and Mn cluster stability. After depletion of PsbO from PSII in in vitro experiments using $CaCl_2$ (*6*) or urea (*7*), O_2 evolution activity is restored to only ∼20% of the control value (intact PSII), and even this low rate requires concentrations of Ca^{2+} and Cl^- higher than those needed by PSII samples depleted of the PsbP and PsbQ polypeptides (*8*). It has been shown that incubation of PsbO-depleted PSII at low Cl^- concentrations causes the loss of two of four Mn atoms from the OEC, but this loss can be prevented by addition of a high concentration of Cl^- (*7, 9*). A recent study of Arg mutants of spinach PsbO showed that the protein is important for retention of Cl^- in the OEC (*10*). Other results indicate that PsbO also plays a role in retention of Ca^{2+}. Ädelroth et al. (*11*) used $^{45}Ca^{2+}$ to show that removal of PsbO either eliminates the Ca^{2+} binding site in PSII or removes a barrier that prevents rapid exchange of Ca^{2+} between the OEC and the surrounding medium. Although PsbO was proposed to be a Ca^{2+} binding protein (*12, 13*), it is acidic (pI 5.2), and therefore, the negative charges on the protein at physiological pH (between 6 and 7) would allow PsbO to bind divalent metal ions nonspecifically.

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[1] Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; DCBQ, 2,6-dichloro-1,4-benzoquinone; DMHA, *N*,*N*-dimethylhydroxylamine; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PsbO, manganese-stabilizing protein; OEC, O_2-evolving complex; PS, photosystem; PSII, photosystem II; RC, reaction center; SDS, sodium dodecyl sulfate; SW-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic proteins; TMACl, tetramethylammonium chloride; UW-PSII, urea- and NaCl-washed photosystem II membranes depleted of PsbO, PsbP, and PsbQ (33, 23, and 17 kDa, respectively) extrinsic proteins.

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Removal of PsbO from the OEC in vivo has different consequences in prokaryotic and eukaryotic PSII. *Synechocystis* sp. PCC 6803 carrying a ΔPsbO mutation is light sensitive, grows slowly under photoautotrophic conditions, evolves O_2 at low rates, and is able to assemble PSII subunits (*14*). Mutant cells of the green alga *Chlamydomonas reinhardtii* lacking PsbO cannot grow photoautotrophically and do not assemble PSII (*15*). In contrast to other higher plants, *Arabidopsis thaliana* contains two PsbO genes. A mutant deficient in PsbO-1 that overexpresses PsbO-2 has significantly altered PSII photochemistry but can grow photoautotrophically (*16*). Suppression of expression of both *A. thaliana* PsbO mRNA's by RNAi causes serious defects in assembly and stability of PSII, and an inability of the transgenic plants to grow photoautotrophically (*17*).

In this work, we report the functional characteristics of PsbO-depleted spinach PSII that was either incubated with a varying concentration of Cl^- or reconstituted with PsbO deletion mutants from spinach that bind in different stoichiometries to the PSII RC. The data on O_2 evolution activity, Ca^{2+} and Cl^- retention, and stability of the Mn cluster show that PsbO does not facilitate Ca^{2+} retention by the OEC in the steady state, nor is it required for the protective effect of Ca^{2+} against reductive inhibition of the Mn cluster in darkness (*18*). In contrast to the result with Ca^{2+}, PsbO has a direct effect on the affinity of Cl^- for PSII (*10*). The data presented here suggest that retention of Cl^- by PsbO is the source of stabilization of the Mn cluster. One copy of PsbO is sufficient to stabilize the Mn cluster under low-Cl^- conditions and to enhance retention of Cl^- in the OEC. However, two copies are needed for optimal Cl^- retention and for maximal O_2 evolution activity. Nonspecific binding of PsbO to PSII has no functional significance.

MATERIALS AND METHODS

N-Terminal Deletion Mutants of PsbO. The recombinant PsbO species (wild type, ΔG3M, ΔR5M, ΔT7M, ΔS13M, ΔK14M, ΔT15M, and ΔE18M PsbO's) characterized in previous studies were used for the experiments presented here. In these mutated PsbO's, the translation initiation codon (Met) replaced the first 3, 5, 7, 13, 14, 15, or 18 amino acid residues in the N-terminal sequence of PsbO. References *19*−*21* give details of the preparation of these mutants.

Preparation of PSII Membranes. Intact PSII membranes [containing PsbO, PsbP, and PsbQ (33, 23, and 17 kDa, respectively) extrinsic proteins], SW-PSII (NaCl-washed PSII membranes depleted of the 23 and 17 kDa extrinsic proteins), and UW-PSII (urea- and NaCl-washed PSII membranes depleted of PsbO, PsbP, and PsbQ extrinsic proteins) were prepared as described by Popelkova et al. (*19*), except that UW-PSII preparations were resuspended for storage in a modified SMN buffer [0.4 M sucrose, 50 mM MES (pH 6), and 100 mM NaCl] to yield reconstitution mixtures having lower Cl^- concentrations.

Reconstitution of UW-PSII with Recombinant PsbO and Activity Assays. The incubation medium used for reconstitution contained 37 mM MES (pH 6), 100 μg/mL BSA, 0.3 M sucrose, and 2% betaine (w/v) (buffer A); betaine was used to prevent aggregation in the reconstitution mixtures, and various concentrations of Ca^{2+} and Cl^- were included depending on the experiment. The Chl concentration was 200 μg/mL, and UW-PSII was incubated with 5 or 10 mol of PsbO/mol of PSII for 1 h at 25 °C in all experiments to ensure optimal reconstitution. Reconstitution stoichiometries were based on PSII reaction center concentrations (0.9 μM for 200 μg/mL PSII) and PsbO concentrations based on the extinction coefficient of the protein (*19, 21*). For experiments that aimed to determine the Cl^- K_M, buffer A contained 10 mM Ca^{2+} and 12.5−17.5 mM Cl^-. In the experiments that aimed to determine the Ca^{2+} K_M, the metal was omitted from buffer A, but 100 mM Cl^- was present. The assay buffers used for measurement of O_2 evolution activity of these reconstituted samples contained 0.4 M sucrose, 50 mM MES (pH 6), 80−100 μg/mL BSA, 600 μM DCBQ as the electron acceptor (buffer B), and various Ca^{2+} and Cl^- concentrations, depending on the experiment. Each assay mixture also included 12−15 μg of Chl. In the assays carried out with varying Cl^- concentrations, O_2 evolution activity was measured at a constant Ca^{2+} concentration (10 mM) and varying concentrations of Cl^- (500−700 μM to 50.7 mM). In the assays with varied Ca^{2+} concentrations, the Cl^- concentration (100 mM) was constant and the Ca^{2+} concentrations ranged from 4 μM to 9.5 mM.

In the experiment designed to study stabilization by Cl^- of the Mn cluster in UW-PSII, samples were incubated in buffer A containing 10 mM Ca^{2+} and either 10, 13, 17, 25, or 100 mM Cl^-. Buffer B, used to determine rates of O_2 evolution, contained 20 mM Ca^{2+} and 100 mM Cl^-. For stability experiments that monitored the loss of Mn^{2+} from SW-PSII, from UW-PSII, and from UW-PSII reconstituted with ΔG3M, ΔS13M, or ΔE18M PsbO (these five samples represented one set) during a 23 h dark incubation period at 4 °C, buffer A contained 10 mM Ca^{2+} and 10 mM Cl^-. Five equivalent sets of samples were prepared. One set was used to assay O_2 evolution activity in buffer B containing 20 mM Ca^{2+} and 100 mM Cl^-, and four other sets were used for Mn quantification by EPR (see Electron Paramagnetic Resonance). In experiments comparing the ability of Ca^{2+} to protect the Mn cluster against reductive inhibition by dimethylhydroxylamine (DMHA), samples were reconstituted with various PsbO's in buffer A containing 40 mM Cl^- in either the presence or absence of 10 mM Ca^{2+}. Aliquots of samples with or without DMHA (10 or 100 mol/mol of PSII) were incubated at 4 °C in darkness. Activity of samples was assayed in buffer B containing 100 mM Cl^- and 20 mM Ca^{2+}. The fraction of centers inhibited by DMHA was calculated as

$$F_I = (A_C - A_R)/A_C$$

where F_I is the fraction of centers inactivated, A_C is the activity of the sample aliquot incubated in the absence of DMHA, and A_R is the activity of the sample aliquot incubated in the presence of DMHA. All O_2 evolution assays were conducted in saturating light at 25 °C.

Electron Paramagnetic Resonance. Manganese quantification was carried out by EPR using a Bruker EMX spectrometer. Each reconstitution mix contained 300 μg of Chl. Four equivalent sets of samples (SW-PSII, UW-PSII, and UW-PSII reconstituted with ΔG3M, ΔS13M, or ΔE18M) were prepared to quantify Mn before and after dark incubation at 4 °C for 23 h. Two sets of samples (incubated for 0 and 23 h) were treated with 50 mM $CaCl_2$ to displace adventitiously bound Mn^{2+} (*22*). Samples were then centrifuged

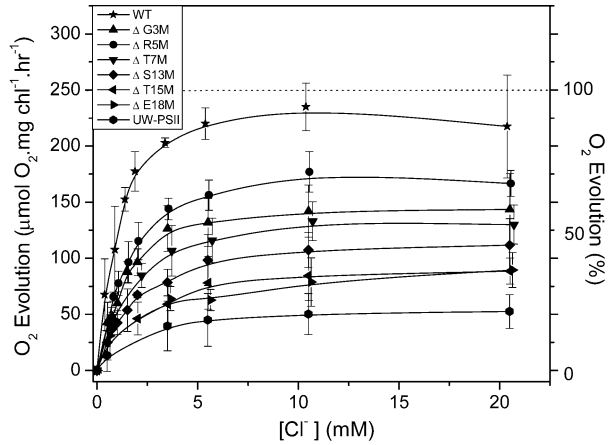

FIGURE 1: Effect of an increasing Cl^- concentration on the O_2 evolution activity of UW-PSII reconstituted with various recombinant PsbO mutants. The dotted line shows maximal SW-PSII activity, and the right axis shows the percentage of that activity. Points are the averages of at least three independent experiments. Error bars correspond to the standard deviation for the particular data point. The Ca^{2+} concentration was held constant at 10 mM, well above the limit for activity saturation by this metal.

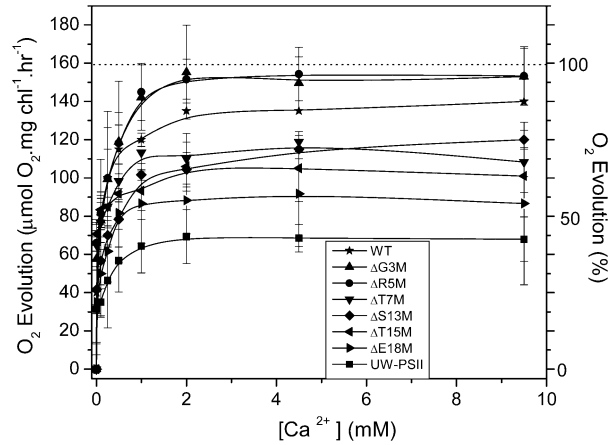

FIGURE 2: Effect of an increasing Ca^{2+} concentration on the O_2 evolution activity of UW-PSII reconstituted with various recombinant PsbO mutants. The dotted line shows maximal SW-PSII activity, and the right axis is a percentage of that activity. Data points are the averages of at least three independent experiments. Error bars correspond to the standard deviation. The Cl^- concentration was held constant at 100 mM.

immediately at 10000*g* for 20 min. The other pair of samples (also incubated for 0 and 23 h) was centrifuged without $CaCl_2$ treatment. Supernatants were discarded, and the pellet was resuspended in 0.6 M HCl to a final volume of 300 μL. Each suspension was loaded into a flat cell and scanned once under the following conditions: microwave power, 200 mW; modulation amplitude, 10 Gpp; modulation frequency, 100 kHz; sweep width, 900 G; time constant, 10 ms; and center field, 3480 G. The microwave frequency was 9.76 GHz. The control (100%) Mn content (~3.5 Mn/250 Chl) was based on the values obtained for SW-PSII samples treated like reconstituted samples.

RESULTS

Effect of PsbO Stoichiometry on the Retention of Ca^{2+} and Cl^-. It has been shown that defective PsbO binding to PSII causes rapid exchange of Cl^- between the OEC and the surrounding medium, as evidenced by an increase in the Cl^- K_M, and that this has a negative effect on O_2 evolution activity (*10*). Deletion mutations in the N-terminal region of PsbO change the binding stoichiometry from two copies (wild type, ΔG3M, and ΔR5M PsbO's) to one copy that assembles normally with high affinity (ΔT7M, ΔS13M, and ΔK14M PsbO's). Further truncations (ΔT15M and ΔE18M PsbO's) are unable to bind effectively to the PSII reaction center (*19*−*21*). To determine the effect of PsbO stoichiometry on the retention of Ca^{2+} and Cl^-, UW-PSII preparations reconstituted with various N-terminal deletion mutants were assayed for activity as a function of the Ca^{2+} or Cl^- concentrations present in the assay buffer. Figure 1 shows the results that were obtained for varied Cl^- concentrations in the presence of a saturating level of Ca^{2+} (10 mM), while Figure 2 presents the results obtained for varied Ca^{2+} concentrations assayed in the presence of a level of saturating Cl^- (100 mM). The ΔG3M and ΔR5M PsbO mutants, each of which rebinds to PSII with a high affinity and a stoichiometry of two subunits per PSII, restore activities with increasing Ca^{2+} and Cl^- concentrations that approach that of wild-type PsbO. In some assays, the activities of ΔG3M and ΔR5M exceed those of wild-type PsbO, but the overlapping error bars at a number of points in Figures 1 and 2 indicate that variations in activity of these three PsbO species are statistically insignificant. In the case of the ΔT7M and ΔS13M mutants, which exhibit high-affinity binding of one copy of PsbO per PSII, the restoration of activities by Ca^{2+} and Cl^- is saturated at a specific activity lower than that observed for wild-type PsbO, and no enhancement of activity is observed with an increase in the concentration of cofactors in the reaction mixture beyond those required to saturate activity. The results obtained with the ΔT15M and ΔE18M mutants, which bind weakly to PSII at approximately one subunit per PSII, exhibit saturation of activity by both Ca^{2+} and Cl^- slightly above the activity of PsbO-depleted PSII (Figures 1 and 2). Determination of the K_M values for both cofactors (Table 1) showed that PsbO stoichiometry has an effect on Cl^-, but not on Ca^{2+} retention under steady-state illumination. For Cl^-, a decrease in the K_M value from 3.9 mM for UW-PSII to 2.2−2.5 mM for UW-PSII reconstituted with ΔT15M or ΔE18M indicates that even weak binding of PsbO to PSII can facilitate Cl^- retention. A significant decrease in the Cl^- K_M to ~1.5−1.6 mM after reconstitution of UW-PSII membranes with ΔT7M or ΔS13M shows that normal binding and functional assembly of the first copy of PsbO to PSII have a significant effect on the retention of Cl^- by the OEC. Binding and assembly of the second copy of PsbO to PSII optimize Cl^- retention; the

Table 1: K_M Values for Cl^- and Ca^{2+} Calculated from the Activity Assays Shown in Figures 1 and 2[a]

sample	Cl^- K_M (mM)	Ca^{2+} K_M (mM)
SW-PSII	0.9	0.15
WT PsbO with UW-PSII	1.0	0.09
ΔG3M PsbO with UW-PSII	1.3	0.15
ΔR5M PsbO with UW-PSII	1.2	0.15
ΔT7M PsbO with UW-PSII	1.5	0.09
ΔS13M PsbO with UW-PSII	1.6	0.15
ΔT15M PsbO with UW-PSII	2.2	0.10
ΔE18M PsbO with UW-PSII	2.5	0.12
UW-PSII	3.9	0.13

[a] SW-PSII, UW-PSII, and UW-PSII reconstituted with recombinant WT PsbO are the control samples.

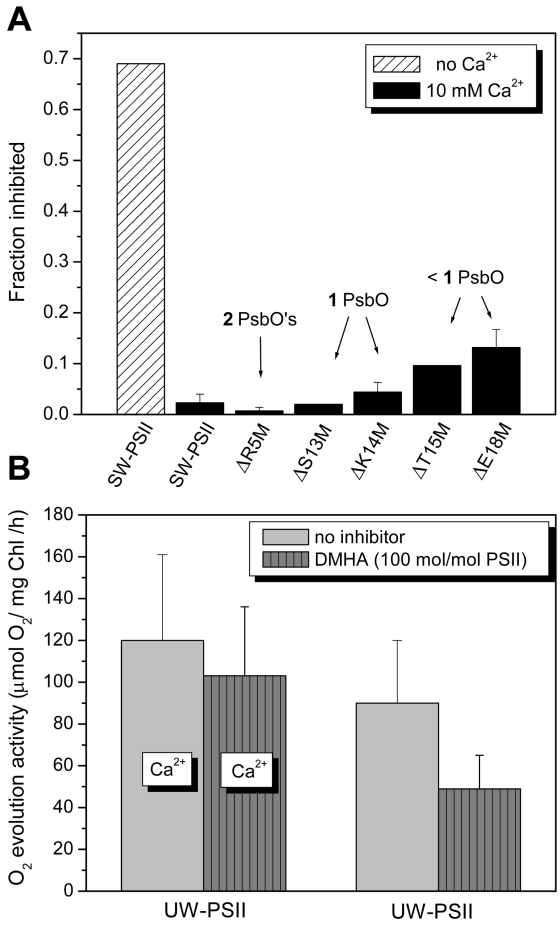

FIGURE 3: (A) Effect of Ca^{2+} on DMHA inhibition of O_2 evolution activity in SW-PSII and PsbO-reconstituted UW-PSII. Samples were incubated in darkness for 24 h at 4 °C. The DMHA concentration of 10 mol/mol of PSII; when present, the Ca^{2+} concentration was 10 mM. (B) Effect of 10 mM Ca^{2+} on the loss of O_2 evolution activity in UW-PSII incubated with or without DMHA (100 mol of DMHA/mol of PSII) for 2 h at 4 °C. Columns depict the averages of two to four experiments.

Cl^- K_M shifts to 0.9−1.3 mM. It should be noted that neither of the truncated proteins that exhibit wild-type activity (ΔG3M and ΔR5M) can restore the Cl^- K_M values to those obtained with either reconstituted or natively bound wild-type PsbO (Table 1). This could be a result of some subtle alterations in the assembly of these mutated PsbO's into PSII caused by the N-terminal truncations.

Protective Effect of Ca^{2+} against Reductive Inhibition. The Ca^{2+} K_M values in Table 1 reveal that PsbO stoichiometry does not affect the affinity of Ca^{2+} for the OEC under steady-state illumination. To explore the effect of PsbO on the ability of Ca^{2+} to protect the Mn cluster against reductant attack and inhibition (*18, 23*), the reactivity of the Mn cluster with dimethylhydroxylamine (DMHA) was characterized using UW-PSII and samples reconstituted with mutated PsbO's that yield different rebinding stoichiometries. Dimethylhydroxylamine is a small reductant ($E^{\circ\prime} = 550$ mV) that reduces up to three of four Mn atoms in Ca^{2+}-depleted SW-PSII, inhibits activity, and releases Mn^{2+} from the OEC (*18*). In agreement with the work of Kuntzleman et al. (*18*), results presented in Figure 3A show that ∼70% of the centers in Ca^{2+}-depleted SW-PSII are sensitive to inhibition by DMHA. In contrast, in the presence of 10 mM Ca^{2+}, the Mn cluster is efficiently protected from the reductant; the fraction of inhibited SW-

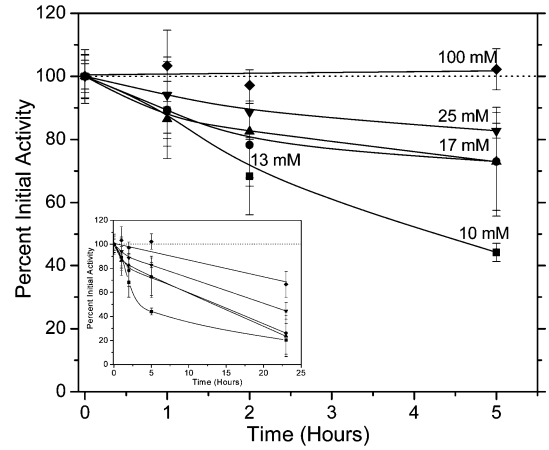

FIGURE 4: Stabilization of UW-PSII activity by Cl^-. Samples were incubated at 4 °C in darkness for up to 5 h. Activities were calculated as a percentage of activity at the beginning of the experiment. Points are the averages of three independent experiments. The inset shows the stabilization of activity of the same samples by Cl^- during a 23 h dark incubation period.

PSII centers is negligible (∼4%). Replacement of native PsbO with recombinant PsbO's [two copies (ΔR5M) or one copy (ΔS13M and ΔK14M)] maintains the protective effect of Ca^{2+} against reductive inhibition of the OEC; the fraction of inhibited centers remains below 5%. Weak binding of recombinant PsbO to UW-PSII (see ΔT15M and ΔE18M in Figure 3A) increases the fraction of inhibited centers to ∼10−13%, indicating that low-affinity binding of PsbO to PSII might alter the ability of Ca^{2+} to block DMHA access to the Mn cluster. To determine whether PsbO binding to PSII facilitates the protective ability of Ca^{2+}, PsbO-depleted PSII was probed in two independent inhibition experiments. The first experiment showed that under conditions similar to those described in the legend of Figure 3A (incubation for 24 h at 4 °C with 40 mM Cl^- with or without 10 mM Ca^{2+}, with or without 10 mol of DMHA/mol of PSII), all UW-PSII samples lose Mn from the OEC; thus, the protective effect of Ca^{2+} is undetectable (data not shown). In the second experiment (Figure 3B), the inhibitor concentration was increased to 100 mol of DMHA/mol of PSII and the incubation time with UW-PSII was shortened to 2 h. The various PsbO-depleted PSII samples were then assayed for O_2 evolution activity. The data (Figure 3B) show that Ca^{2+} at the concentration (10 mM) used here can protect the Mn cluster against reductive inhibition in a manner independent of the presence of PsbO, provided that Mn is not released as a consequence of slow deactivation processes during incubation. An increase in the fraction of inhibited centers, which was observed with ΔT15M and ΔE18M in Figure 3A, was probably caused by a destabilization of the Mn cluster during the 24 h incubation period (see Quantification of Mn Loss by EPR).

Stabilization of the Mn Cluster in UW-PSII by Cl^-. While the data in Table 1 provide evidence that PsbO facilitates Cl^- retention by the OEC in the steady state, the results in Figure 4 show that a high concentration of Cl^- alone is sufficient to enhance Mn retention by the PsbO-depleted OEC in the dark. The results presented in Figure 4 identify the minimum Cl^- concentration needed to stabilize the Mn cluster. In the data shown here, UW-PSII samples were incubated with various concentrations of Cl^- for 23 h at 4

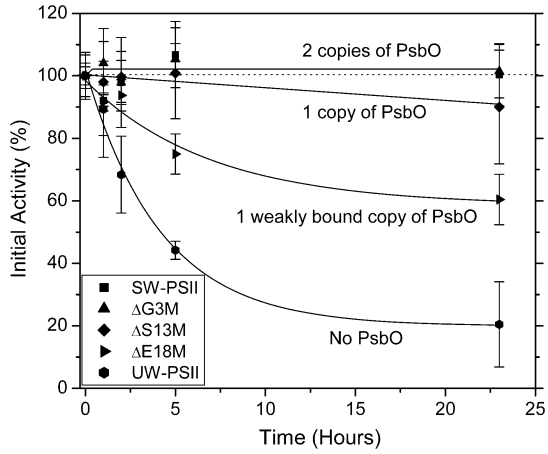

FIGURE 5: Stabilization of O_2 evolution activity of UW-PSII by reconstitution with PsbO truncation mutants. The activity of the each sample is given as a percentage of the initial activity, which corresponds to those shown in Figure 1 for 20 mM Cl^-. The data points are the averages of three independent experiments; data for SW-PSII and UW-PSII are shown as controls.

°C. Loss of Mn was estimated on the basis of the decrease in the initial O_2 evolution activity. The results in Figure 4 show that incubation with 100 mM Cl^- fully stabilizes O_2 evolution activity for 5 h; a sample incubated in 25 mM Cl^- retains ~80% of activity, and 13−17 mM Cl^- samples retain up to 70% of activity after 5 h. A significant decrease in O_2 evolution activity is observed after 5 h with 10 mM Cl^-, when activity declines to 40% of the original value, in agreement with previous experiments by Miyao and Murata (*7*). The inset of Figure 4 depicts the results after incubation for 23 h and shows that the initial activity of UW-PSII decreases to 70% after incubation with 100 mM Cl^-: 25 mM Cl^- protects ~50% of the initial activity, while 10, 13, and 17 mM Cl^- preserve about 20% of the initial activity.

Effect of PsbO Stoichiometry on the Stability of the Mn Cluster. The data on Cl^- stabilization of the OEC defined a minimum Cl^- concentration [10 mM (Figure 4)] that could be used to characterize the effect of PsbO stoichiometry on stability of the Mn cluster. As Figure 5 shows, decreasing the PsbO/PSII stoichiometry also affects the stability of the Mn cluster. In these experiments, UW-PSII membranes reconstituted with various mutated PsbO's in the presence of 10 mM Ca^{2+} and 10 mM Cl^- were incubated at 4 °C in darkness for 23 h and then assayed for activity with 20 mM Ca^{2+} and 100 mM Cl^-. The results reveal that two copies of PsbO (ΔG3M and control SW-PSII) retain initial O_2 evolution activity over the course of 23 h, showing that the Mn cluster remains intact. One copy of PsbO functionally assembled into PSII (ΔS13M) is sufficient to preserve ~90% of the initial activity, as compared to one copy of a mutated PsbO that exhibits weak binding (ΔE18M), where ~60% of the initial activity is retained. The control, UW-PSII lacking PsbO, retains ~20% of the initial activity after incubation for 23 h at 4 °C.

Quantification of Mn Loss by EPR. EPR spectroscopy was used to confirm that the loss of initial activities shown in Figure 5 is due to the loss of Mn from the OEC. The amount of Mn was quantified (Materials and Methods and Table 2) for each sample before and after the 23 h incubation at 4 °C. Four sets of samples were used, and two sets (0 and 23 h incubation) were treated prior to centrifugation with $CaCl_2$ to release any Mn^{2+} that might be weakly bound to PSII (*22, 23*). Samples treated with $CaCl_2$ had a 15% reduction in the absolute Mn concentrations, which supports the conclusion that $CaCl_2$ selectively displaces bound Mn^{2+} (*22, 23*). At the beginning of the quantification experiment, UW-PSII alone and UW-PSII reconstituted with PsbO retained 75% of the Mn atoms compared to the SW-PSII control (see Table 2, 0 h). After incubation for 23 h at 4 °C, UW-PSII lost more than 50% of its initial Mn content. Functional assembly of one or two subunits of PsbO (ΔS13M or ΔG3M, respectively) stabilized the Mn that was present in the OEC at the beginning of the experiment. In contrast, weak binding of PsbO (ΔE18M) preserved only three-quarters of the initial amount of Mn. The data in Table 2 show that the loss of Mn after incubation for 23 h correlates well with the loss of activity observed in the stability assays (see Figure 5).

DISCUSSION

Truncation of PsbO at its N-terminus alters the stoichiometry of binding of the modified proteins to PSII (*19*−*21*). Mutated PsbO's were identified that would rebind to PSII with high affinity, but with stoichiometries of two functional subunits or one functional subunit per PSII RC. Drastic truncations that removed 15 or more amino acids from the N-terminus created proteins that were capable of binding one subunit, but with very low affinity. The experiments presented here utilized some of these mutated PsbO's to probe the effect of its stoichiometry on the retention of inorganic cofactors and to identify a role for each copy of PsbO in the eukaryotic OEC.

Without PsbO and in the presence of low Cl^- concentrations, the Mn cluster in the OEC in darkness is labile and susceptible to relatively rapid reduction and dissociation from PSII as Mn^{2+}. While ~75% of the Mn atoms are retained in freshly prepared UW-PSII (Table 2), approximately half of the metal is lost after incubation for 23 h at 4 °C in darkness. The steady-state Cl^- affinity of PsbO-depleted PSII is also low (see Table 1 and 2, and the schematic model in Figure 6A). In agreement with previous results (*7, 9*), the data of Figure 4 show that binding of the Mn cluster to PSII is stabilized, along with its residual activity, by increasing the Cl^- concentration in a UW-PSII sample during incubation. Table 1 shows, as expected, that the K_M values for Ca^{2+} and Cl^- in extrinsic protein-depleted PSII differ; Ca^{2+} has a higher affinity for the OEC than does Cl^-. Also, in the absence of PsbO, Ca^{2+} protects the Mn cluster against reductive inhibition over relatively long time periods, beyond which the absence of PsbO destabilizes the cluster and Mn^{2+} is released (Figure 3).

The data presented here indicate that PsbO does not affect binding of Ca^{2+} to PSII under steady-state illumination; reconstitution of the protein has no significant effect on Ca^{2+} affinity (Figure 2 and Table 1). In addition, in the dark, PsbO is not required for Ca^{2+} to protect the OEC against reductive inhibition of the Mn cluster (Figure 3 and Table 2) at a concentration (10 mM) where maximum binding of the metal to UW-PSII was detected (*11*). These findings are consistent with structural data that suggest that at least one intrinsic PSII subunit (probably PsbA, the D1 protein) provides the majority of ligands to Ca^{2+} (*24*−*26*).

Table 2: Effect of PsbO Stoichiometry on the Retention of Mn by PSII during Long-Term Incubation[a]

	Mn content						
	0 h incubation		23 h incubation		% initial activity		
sample	with $CaCl_2$	without $CaCl_2$	with $CaCl_2$	without $CaCl_2$	with $CaCl_2$	without $CaCl_2$	no. of PsbO copies per PSII
SW-PSII	100	100	99	98	99	98	2
ΔG3M with UW-PSII	72	84	71	81	99	96	2
ΔS13M with UW-PSII	72	81	74	76	103	94	1
ΔE18M with UW-PSII	66	77	51	62	77	81	1[b]
UW-PSII	80	78	36	37	46	48	0

[a] Samples were incubated in the dark with 10 mM Ca^{2+} and 10 mM Cl^- at 4 °C for 23 h. Samples for Mn determinations were analyzed directly or treated with 50 mM $CaCl_2$ to remove weakly bound Mn^{2+}; all samples were centrifuged at 10000*g* and 4 °C for 20 min and resuspended in 0.6 M HCl. The data are averages of two independent experiments; data deviations are less than 6% for every number. [b] Very weakly bound PsbO subunit.

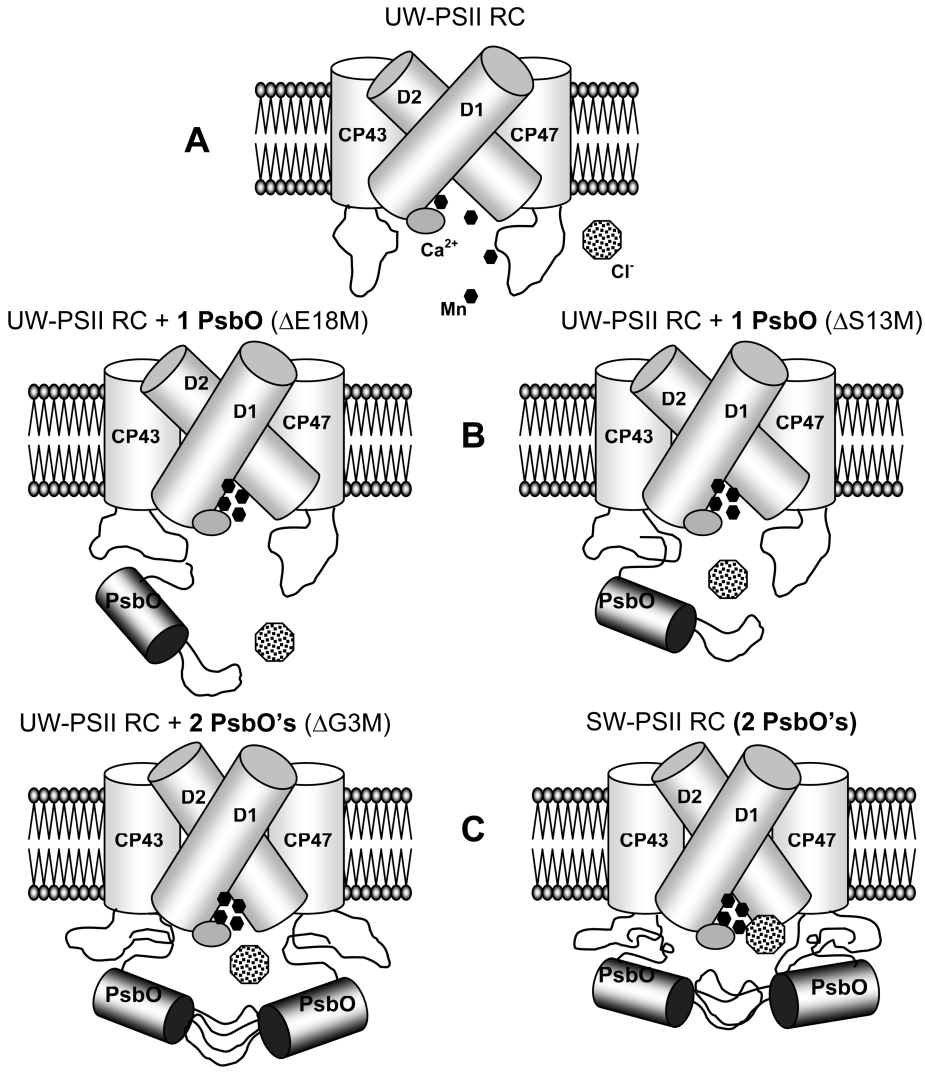

FIGURE 6: Schematic model of the OEC in spinach PSII, summarizing the function of two PsbO molecules with respect to Cl^- cofactor retention. In the steady state, PsbO stoichiometry has no significant effect on OEC Ca^{2+} affinity, and active samples are assumed to retain four Mn atoms per PSII RC. (A) In UW-PSII, the Mn cluster is highly unstable and Cl^- has a very low affinity in the steady state. (B, left) Weak binding of one copy of PsbO (ΔE18M) to UW-PSII increases the PSII Cl^- affinity in the steady state. (B, right) High-affinity binding of one copy of PsbO (ΔS13M) to UW-PSII significantly increases the level of Cl^- retention in the OEC under steady-state illumination. (C, left) Reconstitution of the second copy of PsbO (ΔG3M) into UW-PSII optimizes retention of Cl^- by PSII and significantly enhances steady-state O_2 evolution activity. (C, right) Two copies of native PsbO in salt-washed PSII retain Cl^- in the vicinity of Mn for maximal O_2 evolution activity. Symbols for all the panels: four black dots represent four Mn atoms, the gray oval depicts the Ca^{2+} atom, and the dotted octagon symbolizes the Cl^- atom.

In contrast to the failure of PsbO to enhance the OEC−Ca^{2+} interaction, Cl^- affinity is directly affected by the presence and binding stoichiometry of PsbO under steady-state conditions (Figure 1 and Table 1). It has been shown that Cl^- exchanges with the external medium more rapidly in the higher S states (*27*), so the greater level of protein barriers to Cl^- exchange would be expected to improve retention of the anion in the OEC (Table 1 and Figure 1). These data are also in accord with findings on PsbO Arg mutants that showed that a lower PSII binding affinity was reflected in a stronger Cl^- requirement (*10*) for maximum activity. The results in Figures 4 and 5 and Table

2 show that both 100 mM Cl^- in the absence of PsbO and high-affinity reconstitution of at least one copy of PsbO into PSII in the presence of 10 mM Cl^- can maintain the initial activity of the sample for 23 h. These results, taken together with the fact that PsbO increases the affinity of PSII for Cl^-, indicate that the protein can stabilize the Mn cluster by facilitating retention of Cl^- by the OEC.

Analysis of data on reconstitution of UW-PSII with mutated PsbO's that bind with stoichiometries of one or two subunits per PSII RC revealed that each copy of PsbO has a distinctive function with respect to the Mn and Cl^- cofactors in the OEC. Binding and functional assembly of the first copy of PsbO to PSII ensure full stability and protection of the Mn cluster (see Table 2 and Figures 3 and 5) and significantly increase the level of Cl^- retention in the OEC (see Table 1 and Figure 6B, ΔS13M). Even low-affinity binding of a mutated PsbO defective in its ability to assemble into PSII has some effect on the Mn cluster; the Mn content decreased to ~80% of the initial value, as compared to a decrease of ~50% in PsbO-depleted PSII (see Table 2 and Figure 6B, ΔE18M). Binding of the second copy of PsbO to PSII refines OEC function to generate the highest rates of H_2O oxidation with optimal Cl^- retention (see Figures 1, 2, and 6C). Comparison of Table 1 and Figures 1 and 2 reveals a 20−30% increase in activity, corresponding to a 20−30% decrease in the Cl^- K_M, after reconstitution of the second copy of PsbO into the PSII RC. Addition of Cl^- alone to the samples with one copy of PsbO bound fails to increase activity to the wild-type level (Figure 1); binding of the second copy is required for efficient Cl^- retention in the proximity of the PSII Mn cluster. It is curious that increasing Cl^- concentrations are unable to fully restore activity to the levels of SW-PSII and the reconstituted samples that bind two copies of PsbO. Chloride is known to be required for normal advancement through the S-state cycle from S_2 onward (*28, 29*). If the sample is arrested in the S_2 or S_3 states due to inefficient retention of Cl^- by the OEC, this might cause Q_A^- accumulation and photoinactivation of PSII (*16*). Alternatively, it is possible that an unknown defect in Mn redox activity cannot be repaired by rebinding of a single copy of PsbO.

The mutated PsbO's ΔG3M, ΔR5M, ΔS13M, and ΔT15M also exhibit nonspecific binding of up to four copies of the protein to PSII, as opposed to the ΔT7M and ΔE18M mutants, which exhibit almost no nonspecific binding (*19*−*21*). The data in Figures 1 and 2 and Table 1 reveal that nonspecific binding of PsbO to PSII has no functional significance. This is in agreement with a previous report (*21*) suggesting that three N-terminal sequences (^{1}EGKR^{6}L, ^{8}YDEIQS^{14}K, and ^{16}YL^{18}E) of PsbO are necessary in the wild-type protein to prevent nonspecific binding to PSII, when the protein is present at high concentrations in reconstitution incubation mixtures.

The results reported here on the relationship between PsbO stoichiometry and inorganic cofactor function in the OEC show that each of the two PsbO subunits bound to PSII has a distinct function, a finding that can be reconciled with previous observations of the presence of two copies of PsbO per eukaryotic PSII RC (*19*−*21, 30*−*33*). The first PsbO copy that binds to the OEC stabilizes the Mn cluster and enhances retention of Cl^- in the OEC. The second PsbO copy maximizes O_2 evolution activity by improving efficient retention of Cl^- in the vicinity of the Mn cluster (Figure 6). It is possible that this high-affinity concentrating mechanism for Cl^-, found in plants and algae, is unnecessary in cyanobacteria, which contain only one copy of PsbO per PSII RC (*24*−*26*). In the case of prokaryotes, it is possible that the aquatic environment can provide Cl^- concentrations that are sufficient to maintain high levels of PSII activity. An alternative explanation is that another extrinsic subunit functions as a substitute for the second copy of PsbO, and therefore, removal of PsbO in cyanobacteria does not have the lethal effect on function and assembly of PSII that has been observed in algae or higher plants; PsbV, for example, has been suggested to regulate stability and activity of the OEC in concert with PsbO in cyanobacteria (*34, 35*).

REFERENCES

1. Nelson, N., and Yocum, C. F. (2006) Structure and function of photosystems I and II. *Annu. Rev. Plant Biol. 57*, 521–565.
2. Kashino, Y., Lauber, W. M., Carroll, J. A., Wang, Q., Whitmarsh, J., Satoh, K., and Pakrasi, H. B. (2002) Proteomic analysis of a highly active photosystem II preparation from the cyanobacterium *Synechocystis* sp. PCC 6803 reveal the presence of novel polypeptides. *Biochemistry 41*, 8004–8012.
3. Ghanotakis, D. F., Topper, J. N., Babcock, G. T., and Yocum, C. F. (1984) Water-soluble 17-kDa and 23-kDa polypeptides restore oxygen evolution activity by creating a high-affinity binding-site for Ca^{2+} on the oxidizing side of photosystem II. *FEBS Lett. 170*, 169–173.
4. Miyao, M., and Murata, N. (1985) The Cl^- effect on photosynthetic oxygen evolution: Interaction of Cl^- with 18-kDa, 24-kDa and 33-kDa proteins. *FEBS Lett. 180*, 303–308.
5. Ifuku, K., and Sato, F. (2001) Importance of the N-terminal sequence of the extrinsic 23 kDa polypeptide in Photosystem II in ion retention in oxygen evolution. *Biochim. Biophys. Acta 1546*, 196–204.
6. Ono, T. A., and Inoue, Y. (1983) Mn-preserving extraction of 33-, 23-, and 16 kDa proteins from O_2-evolving PS II particles by divalent salt-washing. *FEBS Lett. 164*, 255–260.
7. Miyao, M., and Murata, N. (1984) Role of the 33 kDa polypeptide in preserving Mn in the photosynthetic oxygen-evolution system and its replacement by chloride ions. *FEBS Lett. 170*, 350–354.
8. Bricker, T. M. (1992) Oxygen evolution in the absence of the 33-kilodalton manganese-stabilizing protein. *Biochemistry 31*, 4623–4628.
9. Kuwabara, T., Miyao, M., Murata, T., and Murata, N. (1985) The function of 33-kDa protein in the photosynthetic oxygen-evolution system studied by reconstitution experiments. *Biochim. Biophys. Acta 806*, 283–289.
10. Popelkova, H., Betts, S. D., Lydakis-Simantiris, N., Im, M. M., Swenson, E., and Yocum, C. F. (2006) Mutagenesis of basic residues R151 and R161 in manganese-stabilizing protein of photosystem II causes inefficient binding of chloride to the oxygen-evolving complex. *Biochemistry 45*, 3107–3115.
11. Ädelroth, P., Lindberg, K., and Andréasson, L. E. (1995) Studies of Ca^{2+} binding in spinach photosystem II using $^{45}Ca^{2+}$. *Biochemistry 34*, 9021–9027.
12. Heredia, P., and De Las Rivas, J. (2003) Calcium-dependent conformational change and thermal stability of the isolated PsbO protein detected by FTIR spectroscopy. *Biochemistry 42*, 11831–11838.
13. Kruk, J., Burda, K., Jemiola-Rzeminska, M., and Strzalka, K. (2003) The 33 kDa protein of photosystem II is a low-affinity calcium- and lanthanide-binding protein. *Biochemistry 42*, 14862–14867.
14. Burnap, R. L., and Sherman, L. A. (1991) Deletion mutagenesis in *Synechocystis* sp. PCC 6803 indicates that the Mn-stabilizing protein of photosystem II is not essential for O_2 evolution. *Biochemistry 30*, 440–446.
15. Mayfield, S. P., Bennoun, P., and Rochaix, J. D. (1987) Expression of the nuclear encoded OEE1 protein is required for oxygen evolution and stability of photosystem II particles in *Chlamydomonas reinhardtii*. *EMBO J. 6*, 313–318.
16. Liu, H. J., Frankel, L. K., and Bricker, T. M. (2007) Functional analysis of photosytem II in a PsbO-1-deficient mutant in *Arabidopsis thaliana*. *Biochemistry 46*, 7607–7613.

17. Yi, X. P., McChargue, M., Laborde, S., Frankel, L. K., and Bricker, T. M. (2005) The manganese-stabilizing protein is required for photosystem II assembly/stability and photoautotrophy in higher plants. *J. Biol. Chem. 280*, 16170–16174.
18. Kutzleman, T., McCarrick, R., Penner-Hahn, J., and Yocum, C. F. (2004) Probing reactive sites within the Photosystem II manganese cluster: Evidence for separate populations of manganese that differ in redox potential. *Phys. Chem. Chem. Phys. 6*, 4897–4904.
19. Popelkova, H., Im, M. M., D'Auria, J., Betts, S. D., Lydakis-Simantiris, N., and Yocum, C. F. (2002) N-terminus of the photosystem II manganese stabilizing protein: Effects of sequence elongation and truncation. *Biochemistry 41*, 2702–2711.
20. Popelkova, H., Im, M. M., and Yocum, C. F. (2002) N-terminal truncations of manganese stabilizing protein identify two amino acid sequences required for binding of the eukaryotic protein to photosystem II and reveal the absence of one binding-related sequence in cyanobacteria. *Biochemistry 41*, 10038–10045.
21. Popelkova, H., Im, M. M., and Yocum, C. F. (2003) Binding of manganese stabilizing protein to photosystem II: Identification of essential N-terminal threonine residues and domains that prevent nonspecific binding. *Biochemistry 42*, 6193–6200.
22. Yocum, C. F., Yerkes, C. T., Blankenship, R. E., Sharp, R. R., and Babcock, G. T. (1981) Stoichiometry, inhibitor sensitivity and organization of manganese associated with photosynthetic oxygen evolution. *Proc. Natl. Acad. Sci. U.S.A. 78*, 7507–7511.
23. Kuntzleman, T., and Yocum, C. F. (2005) Reduction-induced inhibition and Mn(II) release from photosystem II oxygen-evolving complex by hydroquinone or NH_2OH are consistent with a Mn(III)/Mn(III)/Mn(IV)/Mn(IV) oxidation state for dark-adapted enzyme. *Biochemistry 44*, 2129–2142.
24. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) Architecture of the photosynthetic oxygen-evolving center. *Science 303*, 1831–1838.
25. Biesiadka, J., Loll, B., Kern, J., Irrgang, K. D., and Zouni, A. (2004) Crystal structure of cyanobacterial photosystem II at 3.2 Å resolution: A closer look at the Mn-cluster. *Phys. Chem. Chem. Phys. 6*, 4733–4736.
26. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature 438*, 1040–1044.
27. Wincencjusz, H., Yocum, C. F., and van Gorkom, H. J. (1998) S-state dependence of chloride binding affinities and exchange dynamic in the intact and polypeptide-depleted O_2-evolving complex of Photosystem II. *Biochemistry 37*, 8595–8604.
28. Wincencjusz, H., van Gorkom, H. J., and Yocum, C. F. (1997) The photosynthetic oxygen evolution complex requires chloride for its redox state $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions but not for $S_0 \rightarrow S_1$ or $S_1 \rightarrow S_2$ transitions. *Biochemistry 36*, 3663–3670.
29. Wincencjusz, H., Yocum, C. F., and van Gorkom, H. J. (1999) Activating anions that replace Cl^- in the O_2-evolving complex of Photosystem II slow the kinetic of the terminal step in water oxidation and destabilize the S_2 and S_3 states. *Biochemistry 38*, 3719–3725.
30. Xu, Q., and Bricker, T. M. (1992) Structural organization of proteins on the oxidizing side of photosystem II: 2 Molecules of the 33-kDa manganese-stabilizing proteins per reaction center. *J. Biol. Chem. 267*, 25816–25821.
31. Leuschner, C., and Bricker, T. M. (1996) Interaction of the 33 kDa extrinsic protein with photosystem II: Rebinding of the 33 kDa extrinsic protein to photosystem II membranes which contain four, two, or zero manganese per photosystem II reaction center. *Biochemistry 35*, 4551–4557.
32. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1997) Mutation Val235Ala weakens binding of the 33-kDa manganese stabilizing protein of photosystem II to one of two sites. *Biochemistry 36*, 4047–4053.
33. Murakami, R., Ifuku, K., Takabayashi, A., Shikanai, T., Endo, T., and Sato, F. (2005) Functional dissection of two *Arabidopsis* PsbO proteins. *FEBS J. 272*, 2165–2175.
34. Shen, J.-R., and Inoue, Y. (1993) Binding and functional properties of two new extrinsic components, cyt c-550 and a 12-kDa protein, in cyanobacterial photosystem II. *Biochemistry 32*, 1825–1832.
35. Shen, J.-R., Qian, M., Inoue, Y., and Burnap, R. L. (1998) Functional characterization of *Synechocystis* sp. PCC 6803 ΔpsbU and ΔpsbV mutants reveals important role of cyt-550 in cyanobacterial oxygen evolution. *Biochemistry 37*, 1551–1558.

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