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pH Optimum of the Photosystem II H_2O Oxidation Reaction: Effects of PsbO, the Manganese-Stabilizing Protein, Cl^- Retention, and Deprotonation of a Component Required for O_2 Evolution Activity

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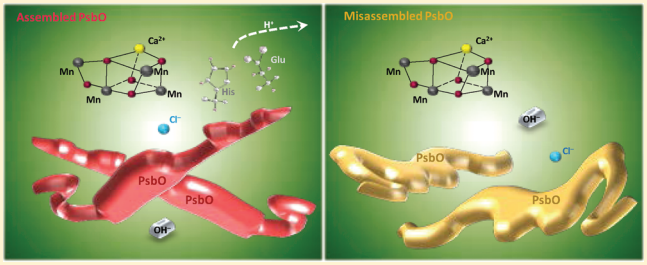
ABSTRACT: Hydroxide ion inhibits Photosystem II (PSII) activity by extracting Cl^- from its binding site in the O_2-evolving complex (OEC) under continuous illumination [Critchley, C., et al. (1982) *Biochim. Biophys. Acta 682*, 436]. The experiments reported here examine whether two subunits of PsbO, the manganese-stabilizing protein, bound to eukaryotic PSII play a role in protecting the OEC against OH^- inhibition. The data show that the PSII binding properties of PsbO affect the pH optimum for O_2 evolution activity as well as the Cl^- affinity of the OEC that decreases with an increasing pH. These results suggest that PsbO functions as a barrier against inhibition of the OEC by OH^-. Through facilitation of efficient retention of Cl^- in PSII [Popelkova, H., et al. (2008) *Biochemistry 47*, 12593], PsbO influences the ability of Cl^- to resist OH^--induced release from its site in the OEC. Preventing inhibition by OH^- allows for normal (short) lifetimes of the S_2 and S_3 states in darkness [Roose, J. L., et al. (2011) *Biochemistry 50*, 5988] and for maximal steady-state activity by PSII. The data presented here indicate that activation of H_2O oxidation occurs with a pK_a of ~6.5, which could be a function of deprotonation of one or more amino acid residues that reside near the OEC active site on the D1 and CP43 intrinsic subunits of the PSII reaction center.



Photosystem II (PSII), the membrane-associated chlorophyll-binding redox enzyme, contains a module called the O_2-evolving complex (OEC) that is the site for oxidation of H_2O. The OEC is part of the PSII reaction center that consists of the core intrinsic proteins: D1 (PsbA), CP47 (PsbB), CP43 (PsbC), D2 (PsbD), and the α and β subunits of cytochrome b_{559} (PsbE and PsbF). The active site of the OEC in eukaryotes is composed of three inorganic cofactors (four Mn atoms, one Ca^{2+} ion, and one Cl^- ion) that are bound by amino acid side chains of D1 and CP43 and surrounded by at least three extrinsic subunits, which are also components of the OEC.[1,2] Although the composition of the extrinsic proteins in PSII varies among different species,[3,4] the largest polypeptide, PsbO or the manganese-stabilizing protein, is found in all prokaryotic and eukaryotic organisms examined so far that employ oxygenic photosynthesis.

The PsbO protein is an intrinsically disordered polypeptide[5] that after initial binding undergoes functional folding and assembly into PSII. Properly assembled PsbO significantly increases the efficiency of the H_2O oxidation reaction; it contributes to retention of the inorganic cofactors in the OEC active site and to the light-driven advancements of the OEC through five distinct redox states, called the S states (S_n, where n = 0−4 and S_1 is the dark-stable state). More details about the structure and function of PsbO are reported in refs 4 and 6−8. The number of PsbO subunits per PSII remains an open question. Current crystallographic models show that prokaryotic PSII possesses a single copy of PsbO, while biochemical experiments with eukaryotes (higher plants) indicate the presence of two subunits of PsbO per reaction center.[8] Investigation of the origins of these differences is continuing. They may arise from the different compositions of PSII extrinsic subunits in cyanobacteria,[4] and/or they may also be a result of the amino acid sequence of PsbO, which exhibits significant differences in eukaryotic and prokaryotic organisms.[6,8]

The Cl^- cofactor plays an essential role in the OEC.[10,11] It has been proposed that it is ligated to Mn[12,13] or Ca^{2+}.[14] Previous studies also showed that OEC-associated Cl^- is required for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transitions,[15,16] and it was also proposed that Cl^- is required for regulation of the redox potential of the Mn cluster.[17] Later studies suggested that Cl^- in the OEC is required to maintain a hydrogen bond network[18] or is involved in the interaction of the OEC with substrate H_2O.[19,20] The results of recent research were interpreted to indicate that the Cl^- ion facilitates the transfer of a proton from the OEC to the lumen.[21−25]

The exact location of Cl^- in the OEC is currently a subject of debate. A number of earlier crystallographic models of cyanobacterial PSII[26−29] do not contain Cl^- in the OEC active

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site, because the electron density of the anion has been difficult to detect in X-ray images from PSII crystals obtained at ≥3.0 Å resolution. Later studies, in which Cl^- was replaced with Br^- or the resolution of X-ray diffraction images was improved, indicated that two Cl^- ions are ∼6−9 Å from the closest Mn atom.[21−23,30,31] Although this distance has to be considered with caution, because the crystals used to produce current X-ray diffraction models of cyanobacterial PSII are susceptible to various amounts of radiation damage due to X-ray dose and temperature,[32] these data improved previous structural models by identifying Cl^- binding sites in PSII. Two Cl^- sites in X-ray diffraction models were interpreted to represent different positions of one Cl^- ion depending on the redox state of the OEC,[31] because these models may correspond to a heterogeneous mixture of Mn oxidation states.[33] Although the presence of Cl^- in multiple sites may not seem to be unusual, given the rapid exchange of the anion with the OEC upon illumination of PSII,[34] it has to be emphasized that the Cl^- ion that is required for O_2 evolution functions at a single site. Graphical analyses of O_2 evolution activity versus Cl^- concentration yield hyperbolic plots and the resulting linear Lineweaver−Burk double-reciprocal plots that are diagnostic for a single binding site that functions in the steady state.[34−36]

The first experimental evidence implicating PsbO in the retention of Cl^- by the OEC showed that its presence decreased the optimal concentration of Cl^- for O_2 evolution activity.[37,38] It was also shown that in the absence of PsbO, increased Cl^- concentrations were required to stabilize the binding of Mn atoms to PSII[39] and that this stabilization of Mn binding by Cl^- could not be mimicked by anions such as sulfate[39] or acetate.[40] These findings were reinforced by the demonstration that PsbO-depleted PSII preparations reconstituted with mutated PsbOs (which either had an altered PSII binding stoichiometry[41] or were defective in assembly into PSII[9,42,43]) exhibit higher Cl^- K_M values than PSII reconstituted with WT PsbO. All of these results are consistent with the current crystallographic models of the cyanobacterial OEC, in which the large flexible loop of the PsbO protein is localized near a Cl^- binding site (see Figure 1 and refs 23 and 30).

The effect of pH on O_2 evolution activity by PSII preparations is to generate a bell-shaped curve; activity increases to a peak between pH 6 and 6.5 followed by rapid declines in activity at assay pH values of ≥7.[35,36,44,45] These declines were interpreted as being a result of deprotonation of carboxyl groups in the vicinity of positive groups that were proposed to bind Cl^-[36] or a consequence of extraction of Cl^- from its functional binding site by OH^-.[12,45,46] Elevated pH has been used as a method for the rapid, reversible depletion of Cl^- from PSII.[34,35,47−49] The question of the nature of the pH-sensitive PSII site inhibited by OH^- was explored in previous studies.[12,50−53] It was found that OH^-, other anions (acetate, F^-, or N_3^-), and primary amines produce inhibitions of water oxidation that are reversed by addition of Cl^-. Many of these inhibitory species function in PSII as Lewis bases that compete with Cl^-, which acts as a Lewis base interacting with a strong Lewis acid, in the case of the OEC perhaps a Mn^{3+} or Mn^{4+} [12] or a site very close to Mn.[54] Thus, the Mn cluster is a possible site of OH^- inhibition of O_2 evolution, although OH^- has not been shown to bind to Mn in the OEC. Because PsbO plays a key role in the retention of Cl^- by the OEC[9,37,38,41−43] and OH^- causes the release of Cl^- from its PSII binding site, which is required for O_2 evolution activity,[36,46] it can be hypothesized that PsbO may play a role in protecting the OEC against

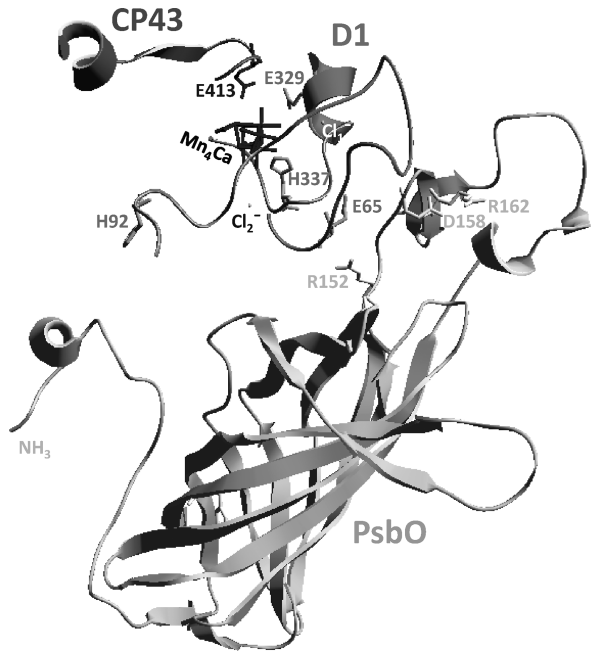

Figure 1. Crystallographic model of the OEC active site from the cyanobacterium *Thermosynechococcus vulcanus* (Protein Data Bank entry 3ARC[30]). The entire PsbO polypeptide (light gray), several domains of the D1 (medium gray) and CP43 (dark gray) proteins, and the Mn_4Ca cluster along with the Cl_1^- and Cl_2^- binding sites (gray dots) are shown. Residues buried in the PSII active site (E65, H92, E329, and H337 in D1 and E413 in CP43) that could possibly be deprotonated at pH 6−7 during cycling of the OEC are also indicated. The N-terminus of PsbO is indicated and starts with Thr4. Residues R152, D158, and R162 in PsbO from *T. vulcanus* are also indicated and are homologous to R151, D157, and R161 in PsbO from spinach.

inhibition by OH^-. To examine this hypothesis, PSII samples that were reconstituted with various PsbO mutants with defined PsbO−PSII binding properties were utilized to assay for O_2 evolution activity as a function of pH and Cl^- concentration. The results indicate that PsbO is important for protection of the OEC against OH^- inhibition with an increasing pH. Under conditions where inhibition by an elevated pH is minimized, activation of O_2 evolution activity by PSII requires deprotonation of a group with a pK_a of ∼6.5, suggesting that protonation and deprotonation of an amino acid residue residing near the Mn_4Ca cluster may be part of a pathway that is required for the transfer of a proton from the OEC to the bulk solvent.

MATERIALS AND METHODS

Photosystem II Preparations. The intact PSII, SW-PSII, and UW-PSII membranes, which were used as control samples, were prepared and stored as described in ref 55. The recombinant PsbO proteins from spinach (WT, ΔG3M, ΔK14M, and ΔT15M) were prepared and characterized as described in refs 55−57. The PsbO-depleted PSII preparations were individually reconstituted with these PsbO proteins to obtain samples with various PsbO−PSII binding stoichiometries, affinities, and specificities. UW-PSII reconstituted with ΔT15M PsbO produced a sample with a single PsbO subunit that is weakly rebound and improperly assembled into PSII specific binding sites.[57] UW-PSII reconstituted with ΔK14M PsbO yielded a sample with a single PsbO subunit that is specifically rebound with a high affinity and correctly folded

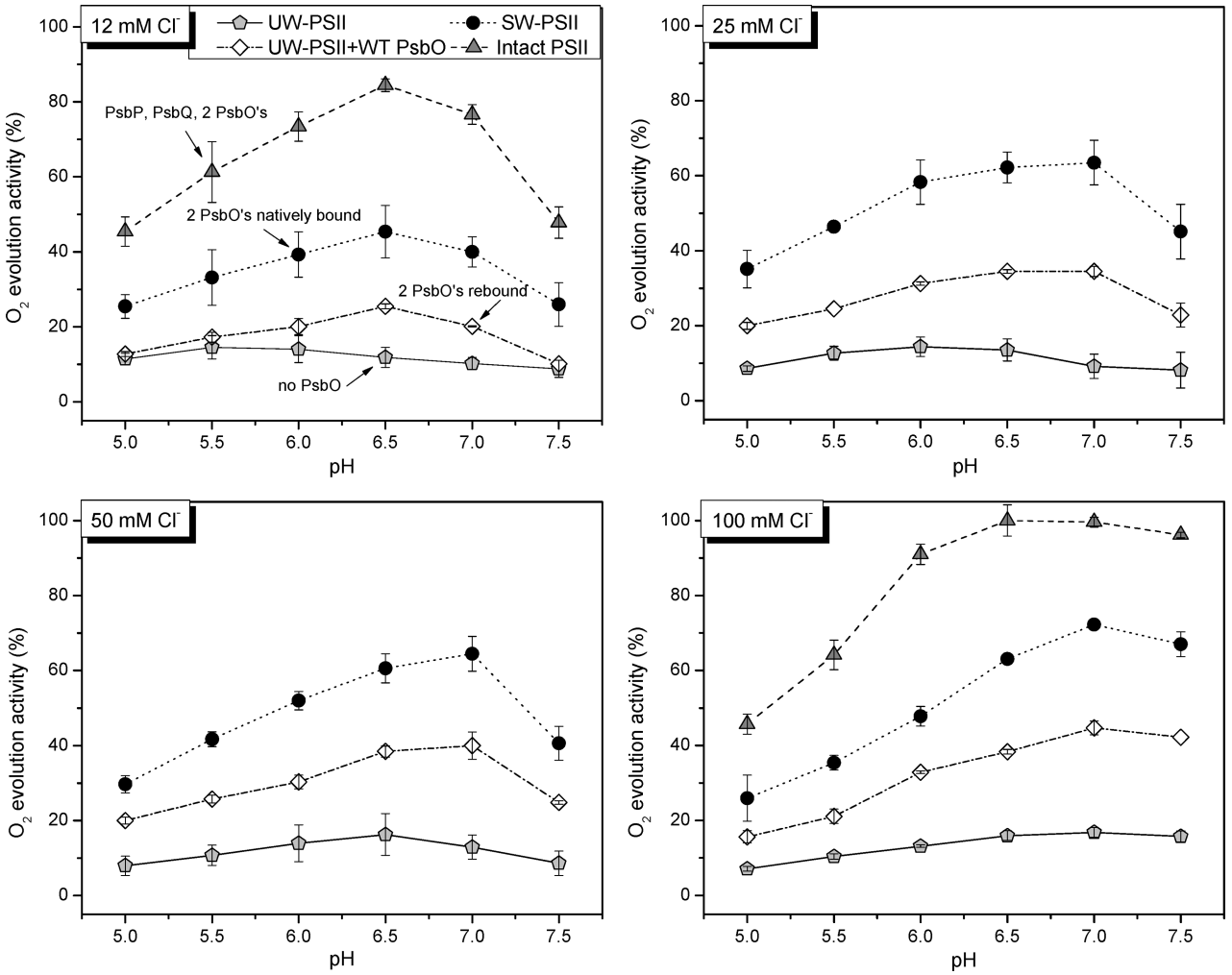

Figure 2. Effect of Cl^- on O_2 evolution activity as a function of pH. PSII, SW-PSII, UW-PSII, and UW-PSII reconstituted with WT PsbO were used. Activities are presented as a function of pH in assay buffer containing 12, 25, 50, or 100 mM Cl^-. UW-PSII was reconstituted with WT PsbO at room temperature for 1 h. Points are the averages, and vertical bars at each point give the standard deviation (n = 2–4). The activity of UW-PSII has not been subtracted from the rates shown for the other samples. For the sake of clarity, the absolute value of maximal activity for intact PSII was set to 100% and the remaining data points are shown as a percentage relative to that value. Control specific activities are given in Materials and Methods.

into PSII.[56] UW-PSII reconstituted with ΔG3M PsbO or WT PsbO produced samples with two PsbO subunits that are specifically rebound with a high affinity and functionally assembled into PSII. UW-PSII reconstituted with the mutated ΔG3M, ΔK14M, or ΔT15M PsbO yielded samples in which the protein is also bound nonspecifically to PSII preparations, in addition to specific binding[55−57] (Table 1 in ref 58).

Reconstitution of UW-PSII with Recombinant PsbO and Activity Assays. The UW-PSII membranes were reconstituted with recombinant PsbO proteins (5 mol of PsbO/mol of PSII to ensure proper binding) for 1 h at room temperature in reconstitution buffer containing 37 mM MES (pH 6), 100 μg/mL BSA, 0.3 M sucrose, 2% betaine (w/v), 10 mM Ca^{2+}, and 17.5 mM Cl^- (for the experiments to determine the Cl^- K_M) or 40 mM Cl^- (to prevent a high Cl^- concentration in the experiments to determine the pH optimum). The Chl concentration in the reconstitution mixtures was 200 μg/mL. Oxygen evolution activity assays were performed in assay buffers containing 0.4 M sucrose, 600 μM DCBQ (used as the electron acceptor), 80−100 μg/mL BSA, and various pH values and concentrations of Ca^{2+} and Cl^- depending on the experiment; 20 mM Ca^{2+}, varying concentrations of Cl^- (0−250 mM), and 50 mM HEPES (pH 7 or 7.5) were used in the experiments to determine the Cl^- K_M, while saturating (5.5 mM) Ca^{2+}, 12, 25, 50, or 100 mM Cl^-, and 50 mM MES or HEPES were used for the experiments to determine the pH optimum at a specific concentration of Cl^- over a pH range from 5 to 7.5. MES was used in assay buffers for the pH range from 5 to 6.5, while HEPES was used for pH 7 and 7.5. For the Cl^- K_M, the averaged activity data points generated hyperbolic saturation curves (as shown in ref 41) that were used for the determination of the Cl^- K_M values based on the Michaelis−Menten equation. Three independent experiments were conducted for each sample to obtain activity data presented here. Data were obtained at six different pH values employing four Cl^- concentrations (12, 25, 50, and 100 mM). Two different native samples (intact and SW PSII) were included along with UW-PSII as a control. Assays were also conducted with four different PSII samples reconstituted with recombinant PsbOs (WT, ΔG3M, ΔK14M, and ΔT15M). To show a significant amount of data clearly along with their interrelations and to control for some sample variability, we set the maximal activity of intact PSII [620 μmol of O_2 h^{-1} (mg of

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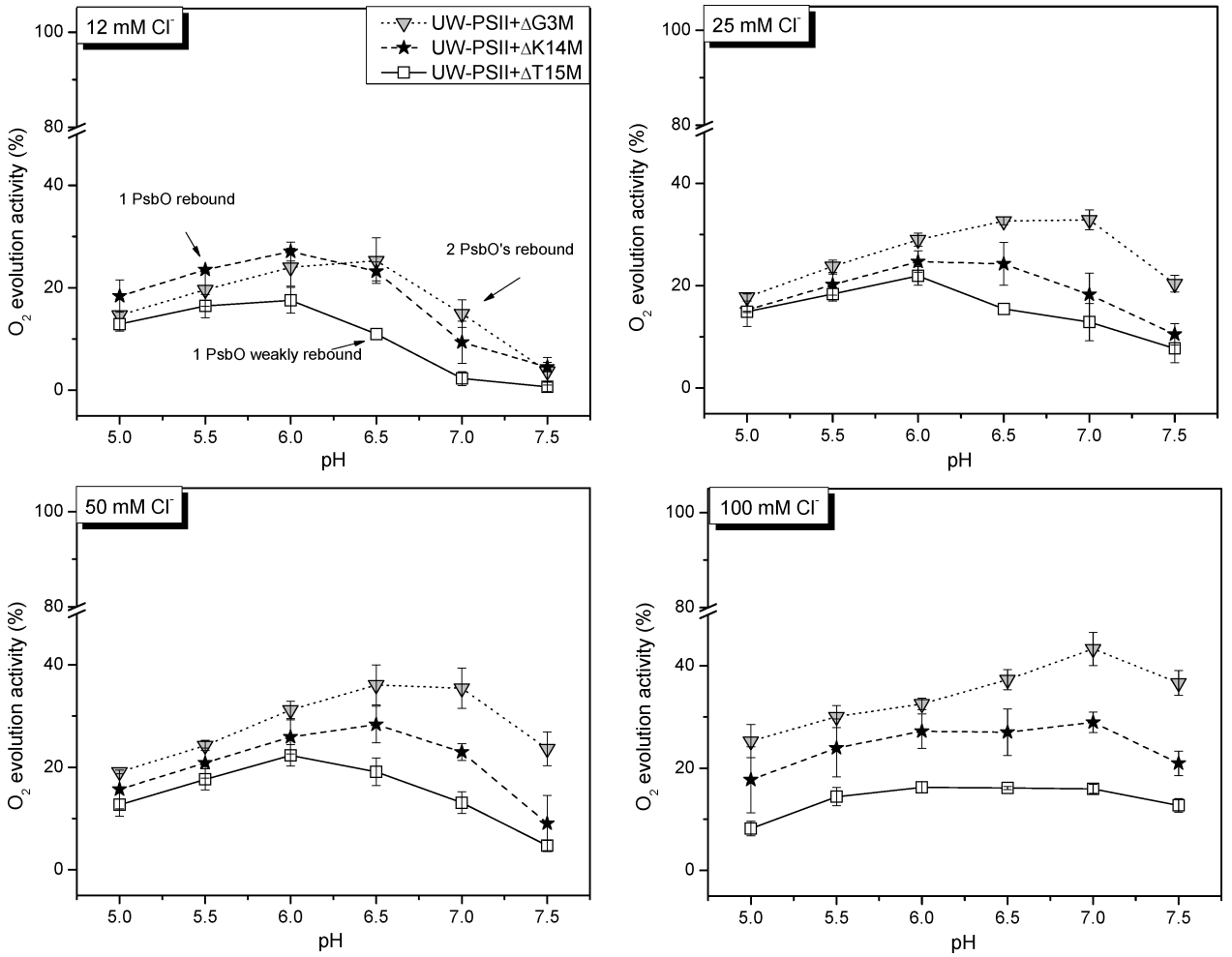

Figure 3. O_2 evolution activity of UW-PSII reconstituted with the PsbO truncation mutants ΔG3M, ΔK14M, and ΔT15M PsbO as a function of assay pH and Cl^- concentration. A break in the *y*-axis has been inserted for the sake of clarity. Other conditions and details are given in the legend of Figure 2.

Chl)$^{-1}$] to 100%, and the remaining activity data points are shown as percentages of this value.

RESULTS AND DISCUSSION

PsbO Protects the OEC against OH^- Inhibition by Promoting the Retention of Cl^- in Its Functional Site in the OEC. To examine the role of PsbO in protecting the OEC against inhibition by OH^-, UW-PSII samples were reconstituted with WT or mutated PsbOs with defined binding properties and assayed for O_2 evolution activity at varying pH values and Cl^- concentrations in assay buffers (Figures 2–4 and Table 1). Figures 2 and 3 present O_2 evolution activities as functions of pH in assay buffers containing 12, 25, 50, or 100 mM Cl^-. The results reveal that the pH optima of samples shown here depend on the concentration of Cl^- in assay buffers as well as on PsbO–PSII binding stoichiometry and affinity. At 12 mM Cl^-, PSII samples with two in vivo bound and assembled PsbO subunits [SW-PSII preparations (Figure 2)] or two in vitro reconstituted subunits [WT PsbO (Figure 2) or the ΔG3M PsbO mutant (Figure 3)] yield a pH optimum of 6.5, while rebinding of a single PsbO subunit with a low (ΔT15M) or high (ΔK14M) binding affinity for UW-PSII produces a pH optimum of 6 (Figure 3). In comparison, a PsbO-depleted PSII sample exhibits an optimum of pH 5.5. Increasing the Cl^- concentration in the assay buffer to 25 mM shifts the pH optima of SW-PSII and UW-PSII reconstituted with WT PsbO or ΔG3M PsbO to a pH range between 6.5 and 7, while reconstitution of UW-PSII with ΔK14M broadens the pH optimum to an interval from pH 6 to 6.5. In the absence of PsbO, UW-PSII exhibits an even broader pH optimum between 5.5 and 6.5. In contrast, PSII reconstituted with ΔT15M PsbO retains a pH optimum of 6 when 25 mM Cl^- is present in the assay buffer. Increasing the Cl^- concentration in the assay buffer to 50 mM produces little change in the pH optimum of samples containing two PsbO subunits (in the range of pH 6.5–7), but it shifts the pH optimum of UW-PSII to a value between 6 and 6.5. The sample reconstituted with ΔK14M PsbO exhibits a pH optimum of 6.5. Weak binding of one copy of ΔT15M PsbO to PSII fails to shift the pH optimum (6) at this Cl^- concentration. A further increase in the Cl^- concentration to 100 mM causes a shift to pH 7 for the optimum of SW-PSII and of PSII reconstituted with either WT PsbO or ΔG3M PsbO. In comparison, the same Cl^- concentration yields a broad optimum between pH 6.5 and 7.5 for PsbO-depleted PSII and between pH 6 and 7 for UW-PSII reconstituted with either ΔK14M or ΔT15M PsbO. For purposes of comparison, Figure 2 also includes data for intact PSII at two, extreme, Cl^- concentrations (12 and 100 mM) to show that the native OEC containing the PsbP and PsbQ polypeptides, in addition to two PsbOs, exhibits results similar

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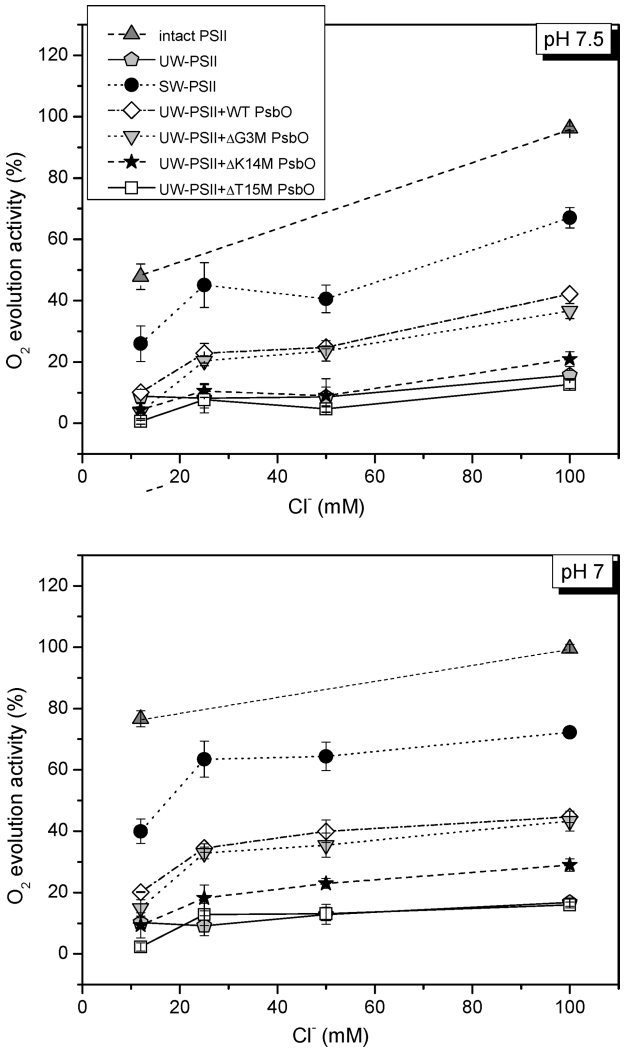

Figure 4. O_2 evolution activity as a function of Cl^- concentration. Percentages of activity at pH 7.5 (top) or pH 7 (bottom) were taken from Figures 2 and 3.

to those obtained for SW-PSII or PSII reconstituted with two PsbO subunits; at 12 mM Cl^-, the pH optimum for intact PSII is 6.5, and at 100 mM Cl^-, the optimum falls between pH 6.5 and 7. Intact PSII and SW-PSII shown here represent identical samples with respect to PsbO that was bound in vivo and assembled into PSII. However, SW-PSII is the comparable control sample for PsbO-reconstituted UW-PSII preparations because it lacks PsbP and PsbQ and undergoes the same treatment with regard to centrifugation and resuspension as UW-PSII preparations.

Table 1. Cl^- K_M Values for SW-PSII, UW-PSII, and UW-PSII Reconstituted with Various PsbO Proteins Measured at pH 6, 7, or 7.5 in Assay Buffer [0.4 M sucrose, 50 mM MES (or HEPES), 20 mM Ca^{2+}, 80−100 μg/mL BSA, and 600 μM DCBQ as the electron acceptor]

sample	Cl^- K_M (mM)		
	pH 6[d]	pH 7	pH 7.5
SW-PSII	0.9	5.9	16.7
UW-PSII with two PsbOs[a]	1.0−1.3	6.1−6.5	27.2−29.9
UW-PSII with one PsbO[b]	1.5−1.6	8.2	51.5
UW-PSII with fewer than one PsbO[c]	2.2−2.5	13.4	77.7
UW-PSII	3.9	13.5	82.8

[a]High-affinity binding of WT PsbO or ΔG3M PsbO. [b]High-affinity binding of ΔS13M PsbO or ΔK14M PsbO. [c]Weak binding of ΔT15M PsbO or ΔE18M PsbO. [d]Data from ref 41 for comparison.

The results in Figures 2 and 3 indicate that at low Cl^- concentrations in assay buffers, samples with binding stoichiometries of fewer than two PsbOs per PSII reaction center exhibit an acidic shift in their pH optima from a value of 6.5, which is the pH optimum for the samples containing two PsbO subunits. As the Cl^- concentration in the assay buffer increases, the pH optima for all of the samples shift toward neutral pH; however the samples containing fewer than two PsbOs per PSII never attain a pH optimum as high as pH 7, nor is O_2 evolution activity restored to the maximal level that is detected in samples that bind two functional copies of PsbO. This suggests that a high Cl^- concentration in the assay buffer cannot fully compensate for the absence of PsbO subunit(s). When present, PsbO in PSII appears to promote optimal retention of Cl^- in the OEC, especially in the higher S states, when the cofactor exhibits fast exchange dynamics with the medium.[34]

The data in Figures 2 and 3 also show that at a saturating concentration (100 mM) of Cl^- in assay buffer, the O_2 evolution activity of the samples as a function of pH exhibits a maximum followed by a small decrease that occurs within the pH range of 7−7.5. It is unlikely that this decline in activity is due to an effect of pH on the reducing side of PSII,[59] because this inhibition reveals itself over a pH range (8.8−9.1) much higher than that used here. It could be argued that this decline in activity might be due to release of PsbO from PSII by a combination of higher pH and increased ionic strength.[60] However, after the activity data for pH 7 and 7.5 from Figures 2 and 3 had been replotted as a function of Cl^- concentration, the results in Figure 4 show that increasing the Cl^- concentration in the assay buffer to 100 mM fails to decrease the O_2 evolution activity of samples. All PsbO variants utilized here exhibit their maximal functional binding to PSII when 5 mol of PsbO/mol of PSII is used in the reconstitution experiment (see refs 55−57). When combined with the results of Kubawara and Murata,[60] who did not observe significant release of extrinsic polypeptides at pH ∼7.5, the result in Figure 4 suggests that the small decrease in activity of samples that is observed within the pH range of 7−7.5 at 100 mM Cl^- is unlikely to originate from either extraction of PsbO from PSII or induction of weakened binding of PsbO to PSII by a high pH accompanied by a high Cl^- concentration in assay buffer. It is possible that the origin is inhibition by OH^-. Although the Cl^- concentration in the assay mixture presented here (∼25−50 mM) greatly exceeds that of OH^- at pH 7 (100 nM), the small hard OH^- ion may compete effectively with the larger, softer Cl^- ion for its binding site in the OEC and inhibit activity at pH ≥7. For example, the pK_a of OH^- is 13.99 at 25 °C;[61] extrapolation of this value to produce an estimated pK_i using the data in ref 62 produces a value for OH^- in the nanomolar range (data not shown). For the pH range where inhibition is observed in this work and ref 36, the nanomolar OH^- concentrations and the concentrations of PSII reaction centers (∼20 nM in ref 36 and 45 nM in this work) in assays could be sufficient to cause the observed partial inhibitions of activity that would be due to a potent inhibitor at low concentrations. Displacement of Cl^- by OH^- has been proposed previously by

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Critchley et al.[46] and Sandusky and Yocum[62] in connection with their characterization of the competition between Cl^- and Lewis bases.[12,50] Homann, on the other hand, interpreted the data in ref 36 to indicate that deprotonation of carboxyl groups in the OEC expels Cl^- by charge−charge repulsion. While the double-reciprocal plots in ref 36 are similar to what would be observed if OH^- were to function as a reversible competitive inhibitor of activity (increasing pH in ref 36 increases K_M without having an effect on V_{max}), the proposal for carboxyl group deprotonation can also explain the loss of Cl^- from PSII with an increasing pH.

The data in Figures 2 and 3 are in agreement with refs 12, 36, and 46 when they show that increasing the Cl^- concentration in the assay buffer from 12 to 100 mM shifts the pH optima toward higher values and weakens inhibition of activity observed in the pH range of 7−7.5 for all of the samples. This increase in activity at higher pH values with an increase in Cl^- concentration is consistent with reversible binding of Cl^-, an essential activator of the oxygen-evolving reaction,[10,11] at the expense of OH^-, a reversible inhibitor of the OEC,[34,35,47−49] at a common site in PSII. It is possible, in theory, that OH^- could act as an inhibitor of PSII reaction centers that would have no effect on the Cl^- K_M but would lower the activity of a sample exposed to an increased pH (noncompetitive inhibition), perhaps by causing the release of Mn atoms. This possibility seems unlikely in light of the data in Figures 2 and 3, where the shifts in the pH optimum also show increased activity for samples with functionally bound PsbO at the higher pH values as the Cl^- concentration is increased, rather than stronger inhibition of activity that would be predicted to occur if OH^- were to function as a noncompetitive inhibitor.

A new finding revealed by the data presented here is that the ability to retain Cl^- and prevent its displacement by OH^- in eukaryotic PSII is clearly facilitated by high-affinity binding of two PsbO subunits per reaction center (see Figures 2 and 3). This observation is reinforced by the Cl^- K_M values at pH 6, 7, and 7.5 for samples with various PsbO:PSII binding stoichiometries and affinities (Table 1); the results for pH 6 were taken from ref 41 for comparison. The data in Table 1 present the relationship among pH, Cl^- K_M, and binding of PsbO to PSII and demonstrate that increasing the pH in the assay buffer increases the Cl^- K_M while increasing the PsbO:PSII binding stoichiometry and affinity decreases the Cl^- K_M. Consistent with data in Figures 2 and 3, the results in Table 1 can be interpreted to indicate that PsbO binding provides significant protection to the OEC against inhibition by OH^- by efficient retention of Cl^-. Interestingly, this key role of PsbO in PSII is not masked by PsbP and PsbQ; the protective effect of Cl^- against OH^- inhibition of the OEC is obvious even with all extrinsic proteins present in the sample, as demonstrated by data with intact PSII (Figure 2). Finally, the increased Cl^- K_M values obtained at higher pH values would be consistent with a competitive interaction between Cl^- and OH^-.

PsbO Facilitates Short Lifetimes of the Higher S States in the Dark by Preventing Inhibition of the OEC by OH^-. The efficient light-driven cycling of the OEC can be correlated with the ability of the higher S states to decay to the S_1 state in the dark. For example, several investigations employing thermoluminescence and O_2 yield measurements revealed that low-activity samples lacking PsbO exhibited unusually long lifetimes for the S_2 and S_3 states in darkness. These results were interpreted to indicate that PsbO facilitates a decay of the higher S states back to S_1 in the dark.[58,63−65] However, the exact mechanism of this function of PsbO is unclear. To obtain insight into this mechanism and to examine its possible connection with the ability of PsbO to prevent inhibition of the OEC by OH^- (see the previous section), we collected the results from four recent reports on various PSII samples with altered PsbO−PSII binding and Cl^- retention properties.[41,43,58,65] These results are shown as two sets of data in Figure 5, where filled circles represent the Cl^- K_M for each

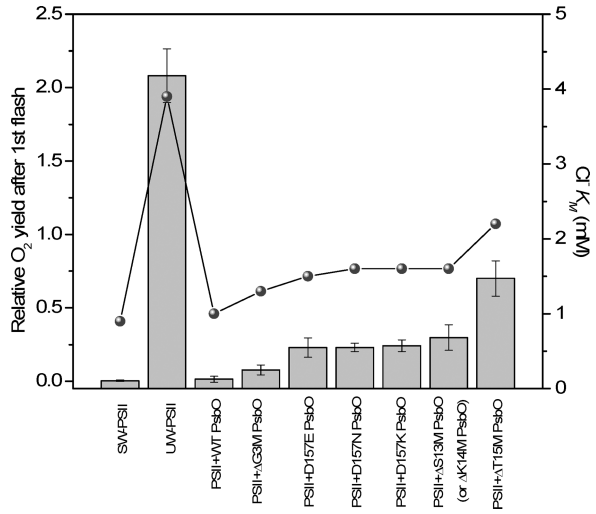

Figure 5. Cl^- K_M values (circles) at pH 6 and O_2 flash yields after the first saturating flash relative to the O_2 yield after the third saturating flash (columns) for SW-PSII, UW-PSII, and UW-PSII reconstituted with wild-type or mutated PsbO. Vertical bars represent the standard deviation. The data were taken from refs 41, 43, 58, and 65. All PSII samples in these references were obtained under very similar reconstitution conditions. Activity assays and O_2 flash yield measurements were conducted in the same assay buffer, except that for the Cl^- K_M determination, the Cl^- concentration ranged between 500 μM and 50.7 mM. For other details, see the text.

particular sample and gray columns depict the corresponding O_2 yields on the first flash. In two of these studies,[58,65] it has been shown that the amplitude of the O_2 yield on the first flash yield correlates with a decrease in PsbO binding affinity and stoichiometry in PSII and is very unlikely to arise from reactions between electrode-generated peroxide and adventitious Mn^{2+}.[58,65] Therefore, it has been suggested that the yield of O_2 on the first flash in various PsbO-reconstituted samples arises from a dark-stable population of PSII centers in the S_2 and S_3 states (see refs 58 and 65). As expected, Figure 5 reveals a positive correlation between the ability of the PSII reaction center to decay rapidly from the higher S states back to S_1 in the dark and the ability of PsbO bound to that center to efficiently retain Cl^- in the OEC. As one can see in Figure 5, samples with low O_2 yields on the first flash (such as SW-PSII or UW-PSII reconstituted with WT PsbO or ΔG3M PsbO) are able to retain Cl^- efficiently, while samples with either high or significantly elevated O_2 yields on the first flash [such as UW-PSII or UW-PSII reconstituted with one copy of PsbO deletion mutants (ΔS13M, ΔK14M, and ΔT15M) or two copies of the Asp157 PsbO mutant] are either ineffective or impaired in their ability to efficiently retain Cl^- in the OEC. Thus, the data in Figure 5 suggest a possible mechanism by which PsbO may facilitate short lifetimes of the higher S states in the dark. The following mechanism may be in use: In the absence of PsbO,

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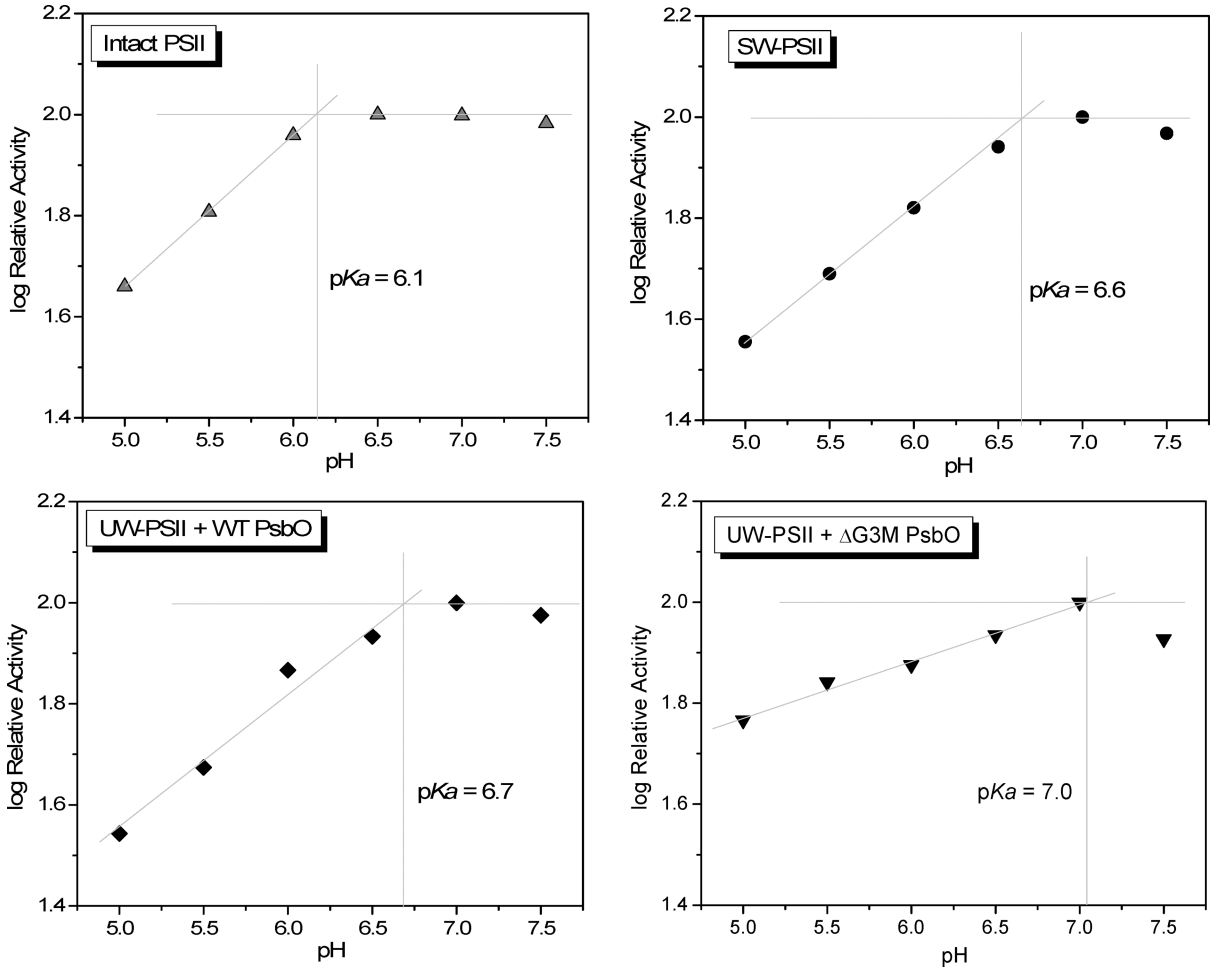

Figure 6. pH dependence of the $\log_{10}$ of relative activities of intact PSII, SW- PSII, and UW-PSII reconstituted with WT PsbO or ΔG3M PsbO. A value of 100 corresponds to the maximal activity of a given sample at 100 mM Cl^- (activities shown in Figures 2 and 3). Graphical extrapolation of the titration curve for each sample shows the pK_a values for intact PSII, SW-PSII, and samples reconstituted with WT and ΔG3M PsbO (6.1, 6.6, 6.7, and 7.0, respectively).

Cl^- is inefficiently retained in the vicinity of the Mn cluster[41] and OH^- causes the loss of Cl^- from its binding site.[12,36,45,46,50] Upon formation of the S_2 or S_3 state, efficient dark relaxation of S_2 and S_3 to S_1 is prevented in the absence of Cl^-. In contrast, the presence of PsbO appears to restrict inhibition of the OEC by OH^- (see the previous section), which in turn may prevent formation of the stable higher states that are unable to decay back to S_1 in the dark (Figure 5 and ref 16).

A positive correlation between the efficient retention of Cl^- in the OEC and a short lifetime of the higher S states in darkness is not unique to spinach PSII preparations (see Figure 5). The results of previous studies of cyanobacterial PSII showed that the OEC in *Synechocystis* sp. PCC 6803 cells carrying the R448S mutation in CP47 was defective in Cl^- retention and exhibited S_2 and S_3 states with unusual stability.[66] The R448 residue resides in the large extrinsic loop E of CP47 that has been proposed in *Synechocystis* sp. PCC 6803 to interact with PsbO through its positive charges at positions R384 and R385.[67] Likewise, the CP43-R305S mutant from *Synechocystis* sp. PCC 6803 exhibited a defect in Cl^- retention along with increased dark stability of the S_2 state, which was interpreted to indicate that the mutant is unable to utilize Cl^- for O_2 evolution activity.[68] R305 is located in the large extrinsic loop E of CP43 along with E352; mutation of the latter residue creates a defect in PsbO binding in *Synechocystis* sp. PCC 6803.[69] All of these results for *Synechocystis* sp. PCC 6803 indicate that both the CP47-R448S and CP43-R305S mutations in cyanobacterial cells disrupt the interface between PsbO and the PSII intrinsic CP43 and CP47 polypeptides that is important for efficient retention of Cl^- by PSII (see ref 11). As a result, the cyanobacterial R448S and R305S mutants experience defects in Cl^- retention. The absence of Cl^- in its binding site leads to formation of dark-stable states of the OEC[16] in *Synechocystis* sp. PCC 6803, by analogy to the situation described above for spinach PSII preparations. Taken together, the results presented in Figure 5 and refs 66 and 68 would suggest that the mechanism by which PsbO prevents inhibition of the OEC by OH^- and promotes a short lifetime of the higher S states in darkness may be similar in all organisms (prokaryotic and eukaryotic) that employ oxygenic photosynthesis.

Deprotonation of an OEC Component Is Required for Maximal Rates of H_2O Oxidation. Additional analyses of the data obtained at 100 mM Cl^- on PSII samples containing two PsbO subunits that are either assembled in vivo [intact PSII and SW-PSII (Figure 2)] or functionally reconstituted in vitro [WT and ΔG3M PsbO (Figures 2 and 3)] into PSII membranes revealed another factor that is essential for efficient

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H_2O oxidation in PSII. To analyze the data, we determined the relative values of O_2 evolution activity for the samples [a (relative) activity of 100 corresponds to the maximal activity of the sample at 100 mM Cl^-] and plotted the logarithms of these values as a function of pH (Figure 6).[70] It should be noted that a high (~100 mM) concentration of Cl^- in the assay buffer combined with the presence of two PsbO subunits per PSII ensures the efficient retention of Cl^- near the Mn cluster, which in turn allows for maximal O_2 evolution activity by PSII (see refs 41 and 43). Maximal activity is an indication of effective interference by Cl^- with OH^- inhibition. As one can see in Figure 6, the activity data produce titration curves with an apparent pK_a between 6.1 and 7.0. Application of the same method[70] to evaluate the data obtained for thylakoids or PSII preparations in earlier studies produced an apparent pK_a for activation of O_2 evolution activity in a similar interval between 5.9 and 7.1.[35,45,46,71] In theory, these pK_a values suggest the deprotonation of an amino acid residue in the activity of an enzyme. It could be that this residue is His, because the estimated usual range of pK_a values for His in proteins is 6–7, while the corresponding ranges of values for other amino acid residues in proteins are normally either lower (3.5–4.5 for Asp and Glu) or higher (10.4–12 for Lys and Arg).[72] However, the normal pK_a value of an amino acid residue can be shifted from its native value by several pH units when it is buried in the core of a protein and experiences strong charge–charge interactions;[73−78] Glu, Asp, Lys, and Cys buried in enzymes were found to exhibit shifts from their normal pK_a values to values between 6 and 7.[73−75,78] Thus, Figure 6 shows that optimal O_2 evolution activity of PSII probably requires deprotonation of one or more residues residing in a proton transfer network between the OEC active site and the lumen,[79,80] but because of the electrostatic influence of charged groups that are present in the OEC active site, the identity of that residue(s) cannot be unequivocally determined from the results presented here. The most recent crystal structure of PSII[30] shows several buried residues in the PSII active site (E65, H92, E329, and H337 in D1 and E413 in CP43) that might participate in the deprotonation process during cycling of the complex (see Figure 1).

The results presented here are summarized schematically in Figure 7. An efficient H_2O oxidation reaction by PSII requires deprotonation of a residue in the vicinity of the Mn_4Ca cluster along with the presence of two fully assembled PsbO subunits that restrict OH^- inhibition of the OEC by retaining Cl^- in its OEC binding site (see Figure 7A). The presence of one or two misassembled PsbO subunits in eukaryotic PSII fails to retain Cl^- efficiently in the OEC, which in turn facilitates the loss of Cl^- from its PSII binding site as the pH is increased (see Figure 7B). The relations among Cl^-, OH^-, and PsbO in the presence of only one correctly folded PsbO subunit (ΔK14M) can also be represented by this model (Figure 7B).

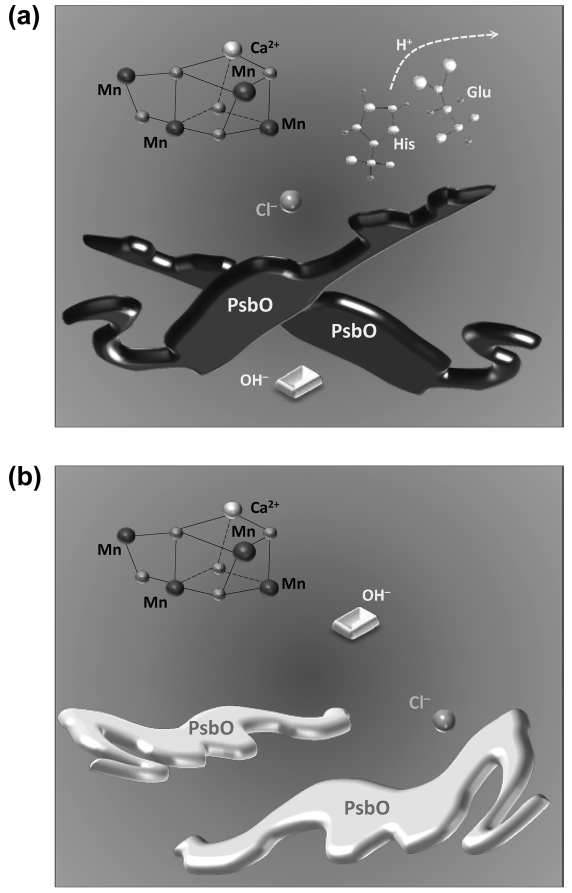

Figure 7. Schematic model for the role of PsbO in the prevention of inhibition of the OEC by OH^- and for the presence of a residue required for H^+ transfer in eukaryotic PSII. The PsbO subunits are shown along with the Mn_4Ca cluster, the Cl^- cofactor, the OH^- ion, and some of the residues capable of deprotonation in proteins at pH 6–7. (A) In a highly active PSII sample containing two correctly assembled PsbO subunits (dark gray), PsbO functions as a barrier against OH^- inhibition of the Mn_4Ca cluster by retaining Cl^- efficiently in its OEC binding site. Deprotonation (indicated by the dashed arrow) of His, Glu, or other residues residing near the Mn atoms is required for efficient cycling of the OEC. (B) The presence of one or two misassembled subunits of PsbO (light gray) in eukaryotic PSII fails to retain Cl^- efficiently in the proximity of the Mn cluster and facilitates the access of OH^- to the OEC. The loss of Cl^- from the PSII active site leads to a strongly but reversibly inhibited PSII sample.

CONCLUSION

Eukaryotic PSII binds a single Cl^- atom,[81] consistent with double-reciprocal plots ($1/V$ vs $1/[S]$) of Cl^- activation of H_2O oxidation that show one functional site.[34−36] At pH >7, Cl^- is lost from the OEC. The results presented here are consistent with the conclusion that a key role of PsbO in PSII is to function to protect the OEC against inhibition by OH^-. Through facilitation of efficient retention of the single Cl^- atom in eukaryotic PSII, PsbO prevents inhibition of the OEC by OH^-. This PsbO function in turn ensures a short lifetime of the S_2 and S_3 states in the dark and allows for maximal cycling of the OEC under continuous illumination. The data reported here also show that O_2 evolution is activated at a pK_a of ~6.5, suggesting that deprotonation of one or more amino acid residues residing near the OEC active site is required for efficient H_2O oxidation. Consistent with this suggestion, recent studies employing mutagenesis or computations based on the PSII crystal structure proposed that several buried histidine (D1-His92 and D1-His337), glutamate (D1-Glu65, D1-Glu329, and D2-Glu312), or aspartate (D1-D59 and D1-D61) residues near the OEC could be involved in putative channels that transport protons from the OEC to the lumen.[79,80,82]

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Notes

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■ ABBREVIATIONS

BSA, bovine serum albumin; DCBQ, 2,6-dichloro-1,4-benzoquinone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PsbO, manganese-stabilizing protein; OEC, O_2-evolving complex; PS, Photosystem; SW-PSII, NaCl-washed Photosystem II membranes depleted of 23 and 17 kDa extrinsic proteins; Tris, tris(hydroxymethyl)aminomethane; UW-PSII, urea NaCl-washed Photosystem II membranes depleted of PsbO, PsbP, and PsbQ (33, 23, and 17 kDa, respectively) extrinsic proteins.

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