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The PSII calcium site revisited

M. Miqyass · H. J. van Gorkom · C. F. Yocum

Abstract Oxidation of H_2O by photosystem II is a unique redox reaction in that it requires Ca^{2+} as well as Cl^- as obligatory activators/cofactors of the reaction, which is catalyzed by Mn atoms. The properties of the binding site for Ca^{2+} in this reaction resemble those of other Ca^{2+} binding proteins, and recent X-ray structural data confirm that the metal is in fact ligated at least in part by amino acid side chain oxo anions. Removal of Ca^{2+} blocks water oxidation chemistry at an early stage in the cycle of redox reactions that result in O-O bond formation, and the intimate involvement of Ca^{2+} in this reaction that is implied by this result is confirmed by an ever-improving set of crystal structures of the cyanobacterial enzyme. Here, we revisit the photosystem II Ca^{2+} site, in part to discuss the additional information that has appeared since our earlier review of this subject (van Gorkom HJ, Yocum CF In: Wydrzynski TJ, Satoh K (eds) *Photosystem II: the light-driven water:plastoquinone oxidoreductase*), and also to reexamine earlier data, which lead us to conclude that all S-state transitions require Ca^{2+}.

Keywords Calcium · Photosystem II · Oxygen evolution · S-states · Thermoluminescence

Abbreviations

Chl	Chlorophyll
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N', N'-tetraacetic acid
EXAFS	Extended X-ray absorption fine structure
FT-IR	Fourier transform infrared
OEC	Oxygen-evolving complex
PS	Photosystem
PsbO	The 33 kDa extrinsic protein
PsbP	The 23 kDa extrinsic protein
PsbQ	The 17 kDa extrinsic protein
TL	Thermoluminescence
XANES	X-ray absorption near-edge structure

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Introduction

Calcium occupies a prominent position in metallobiochemistry research on account of its abundance in living systems and the diversity of biochemical processes in which it is a participant. A divalent alkaline earth, Ca^{2+} is ordinarily found in biological systems with six to seven ligands, many of which are oxygens (peptide backbone carbonyls and carboxyl oxygens of proteins, H_2O (Kretsinger and Nelson 1976)). As such, it accommodates itself to binding sites in a number of enzymes (proteases, lipases, nucleases, for example) where it contributes to stabilization of protein structure, and is, in some cases, essential for catalytic activity. The signaling function of Ca^{2+} is also essential as an intracellular "second messenger" that transduces signals received by the cell exterior

(Bootman and Berridge 1995). In this system, the reversible binding of Ca^{2+} (K_d = ~10^{-6}–10^{-7} M) to EF hand proteins (calmodulins, troponin C, calbindin, and so on (Strynadka and James 1989; Lewit-Bentley and Rety 2000)) provides a sensitive monitoring system, that couples cellular metabolism to signals arriving at the cell surface. Unfortunately, these functions and the wealth of structural and functional data derived from them provide little in the way of comparative information that can be used to sort out another major function of Ca^{2+}, that of an essential cofactor of the inorganic ion complex (4Mn, Ca^{2+}, Cl^-) in PSII that oxidizes H_2O to O_2. So far as we can say, this is the only redox reaction that is known to require Ca^{2+} as a cofactor, but it is also important to bear in mind, that this is the only redox reaction in the biosphere that oxidizes H_2O to O_2.

Photosystem II (PSII) contains a set of intrinsic membrane proteins (PsbA, B, C, D, E, and F) that appear to function either directly or indirectly as the ligation sites for the organic and inorganic components of the electron transfer chain (Eaton-Rye and Putnam-Evans 2005; Nixon et al. 2005). It also contains a number of small peptides that are involved in assembly and stabilization of the multisubunit complex (Thornton et al. 2005). Photosystem II is somewhat an unusual membrane protein complex in that it contains three tightly-bound extrinsic proteins. In eukaryotes, these are PsbO (the manganese stabilizing protein), PsbP (the 23 kDa polypeptide) and PsbQQ (the 17 kDa polypeptide); in prokaryotes, PsbP and PsbQ are replaced by U and V (cytochrome c550) (Seidler 1996; Burnap and Bricker 2005). Early experimentation to unravel the structure and function of the components of PSII showed that extraction of PsbP and PsbQ from spinach PSII preparations results in a strong inhibition of steady state O_2 evolution activity. Intensive efforts to understand why reconstitution of the polypeptides alone would not restore activity led to the discovery (Ghanotakis et al. 1984a; Miyao and Murata 1984) that Ca^{2+} would restore activity. This, in turn, has spawned a number of efforts in many laboratories to understand the role of Ca^{2+} in the structure and function of the OEC. At the present time, there are about 300 publications on Ca^{2+} that are related to its role in PSII, which testifies to the robust interest in this phenomenon. Here, we revisit the PSII Ca^{2+} site in part to take account of the observations that have appeared since we completed and submitted our last review on the subject (van Gorkom and Yocum 2005).

The basics: extraction, reconstitution, stoichiometry, site specificity

There are two methods for extraction of Ca^{2+} from PSII, with differing structural consequences. The first method involves exposure of the intact enzyme to high ionic strength (1–2 M NaCl) (Ghanotakis et al. 1984a; Miyao and Murata 1984), which generates a PSII sample that lacks PsbP and Q, and that exhibits low activity in steady state assays. The highest extent of activity reconstitution is produced by Ca^{2+} addition to these samples. It is generally agreed that among all other metals tested, only Sr^{2+} is capable of reconstituting O_2 evolution activity, but at lower rates (Ghanotakis et al. 1984a; Boussac and Rutherford 1988b). Lockett et al. (1990) reported that VO^{2+} could also replace Ca^{2+} in restoration of O_2 evolution activity and formation of the S_2 multiline signal. So far as we can determine, there have been no further experiments on this phenomenon. The original procedure for Ca^{2+} extraction has been amended to include illumination with continuous room light, which accelerates the rate of activity loss (Miyao and Murata 1986), and inclusion of a chelator (1 mM EDTA or EGTA) is recommended to suppress the high concentrations of adventitious Ca^{2+} found in PSII preparations (Ghanotakis et al. 1984a). The effect of illumination on Ca^{2+} release from the OEC was in fact first noted by Dekker et al. (1984), who exposed salt-washed PSII preparations to single-turnover flashes, and found that the 200 μs decay phase of the $Q_A^- P680^+$ recombination reaction increased in amplitude as a function of flash number, out to about 150 flashes; the original amplitude was restored by Ca^{2+} addition. Boussac and Rutherford (1988a) illuminated intact PSII preparations with single turnover flashes and then exposed the samples to high ionic strength before assays of activity. The results showed that, among the S-states of the redox cycle in H_2O oxidation, the S_3 state was most susceptible to activity loss due to Ca^{2+} extraction. Variations on this method include the procedure described in Kalosaka et al. (1990) in which intact PSII samples are exposed to lower pH (5) during high salt treatment in darkness, and isolation procedures (Ikeuchi et al. 1985; Ghanotakis and Yocum 1986) that remove LHCII and PsbP and PsbQ to produce PSII samples that are Ca^{2+} depleted.

In steady state assays of activity, salt-washed PSII samples respond to added Ca^{2+} within the dead time of a Clark electrode (<5–6 s), and the reconstituted activity is sensitive to inhibition by EDTA. These

observations indicate that, in the absence of the extrinsic subunits, the OEC Ca^{2+} site is open to the external medium, and that Ca^{2+} exchanges freely and rapidly with the site. The literature on Ca^{2+} affinity of salt-washed PSII preparations reports a wide range of K_d or K_M values for the metal (van Gorkom and Yocum 2005), from low (μM) to 1–2 millimolar concentrations. In the case of steady state assays of activity, this may be a consequence of differences in sample treatment (Han and Katoh 1995) and possibly to the presence of modified reaction centers whose Ca^{2+} affinities have been altered by the extraction treatment (Han and Katoh 1995). It is also probable that the values of Ca^{2+} affinity are affected in such experiments by the initial presence of other metals in the Ca^{2+} site at the start of the assay or their binding in higher S-states during the assay.

The alternate method of Ca^{2+} extraction was developed by Ono and Inoue (1988), who showed that brief exposure of intact PSII to pH 3 citrate solutions resulted in a substantial loss of activity, but the inhibited samples retained PsbP and Q. For these samples, reconstitution of activity required long term incubation in the presence of Ca^{2+} prior to activity assays. The presence of the PsbP subunit presumably blocks rapid access of Ca^{2+} to its binding site in the OEC (Ghanotakis et al. 1984b; Miyao and Murata 1986; Ono and Inoue 1988), and this in turn makes it difficult to assess the actual Ca^{2+} affinity of the site in this preparation. Recent experiments probing acid-treated PSII with reductants showed that access to the Mn cluster by NH_2OH was increased relative to that of a larger reductant (hydroquinone), and that Ca^{2+} reconstitution closed this pathway (Vander Meulen et al. 2002; 2004). In addition, it was shown that room temperature (25°C) reconstitutions of the Ca^{2+} site proceeded more rapidly than at 4°C, and that the presence of a non-activating divalent metal (Mg^{2+}) along with Ca^{2+} significantly decreased the concentration of the latter metal that was required to reconstitute activity; the K_d in this case was estimated to be about 6 μM (Vander Meulen et al. 2004). It was proposed that the non-activating metal, combined with a higher incubation temperature, may have weakened the binding of the extrinsic subunits sufficiently to permit facile access of Ca^{2+} to its binding site in the OEC. Alternatively, it is possible that Mg^{2+} ions bound to sites outside the OEC decreased the concentration of Ca^{2+} needed to reconstitute activity.

These observations suggest that the intrinsic affinity of Ca^{2+} for PSII is very high, and that treatment with high ionic strength (1–2 M NaCl) may alter the affinity. If PSII is exposed to a much lower ionic strength (50 mM $Na_2SO_4^{2-}$ at pH 7.5 (Wincencjusz et al. 1997)), PsbP and PsbQ are released, but the resulting PSII sample retains Ca^{2+} and exhibits high rates of O_2 evolution activity when only Cl^- is added back to the assay buffer. This result indicates that Ca^{2+} binding to the OEC depends on factors other than the presence of PsbP and Q. Nevertheless, Barra et al. (2005) have carried out heating experiments at 47°C in which a loss of O_2 evolution activity is shown to correlate well with the loss of the PsbQ subunit. Comparison of the fluorescence properties of heated samples with those of samples depleted of Ca^{2+} (by citrate exposure) or of both Mn and Ca^{2+} (by Tris treatment) prompted the authors to propose that loss of the PsbQ subunit, rather than PsbP, is accompanied by loss of Ca^{2+}. This is an interesting possibility. The ability of the authors to generate the S_2 multiline signal upon illumination at 200 K would, for other PSII samples, be indicative of the presence of Ca^{2+} in its native binding site (Boussac et al. 1990; Ono and Inoue 1990a). It should also be mentioned that the biosynthetic incorporation of Sr^{2+} into the OEC (Boussac et al. 2004), which was accomplished by culturing T. elongatus in growth medium where Ca^{2+} was replaced by Sr^{2+}. The resulting PSII preparations exhibited the spectroscopic signatures expected for this substitution (see below).

The stoichiometry of Ca^{2+} in the OEC was first estimated to be about 1/PSII on the basis of elemental analyses of biochemically resolved preparations (Shen et al. 1988). A second Ca^{2+} atom was found to be tightly bound to antenna proteins (Han and Katoh 1993), probably CP29 (Jegerschöld et al. 2000). Binding of Ca^{2+} has also been examined under static (dark) conditions with an ion-specific electrode (Grove and Brudvig 1998). High affinity ($K_d = 1.8$ μM) binding of about four Ca^{2+}/PSII was detected in intact preparations, ascribed to sites associated with LHCII. Even higher affinities (0.05–0.19 μM) were found for preparations depleted of Ca^{2+}, extrinsic polypeptides, or Mn. These affinities are assigned binding to the OEC Ca^{2+} site or to Mn binding sites in case of preparations depleted of this metal; in the case of Ca^{2+} depleted PSII, a stoichiometry of 2–3 Ca^{2+}/OEC was found. This stoichiometry was reexamined using $^{45}Ca^{2+}$ by Ädelroth et al. (1995), who found about 1 Ca^{2+}/OEC. These investigators also used $^{45}Ca^{2+}$ to characterize the exchange properties of Ca^{2+} in dark-adapted PSII. The results are somewhat different from those obtained with the steady-state assays. For acid-treated PSII, two binding sites were detected with apparent K_d values of 0.06 and 1.7 mM. Calcium binding to this preparation was slow (about 10 h were required to reach a maximum level of metal binding and activity, and release of

the labeled ion was similarly very slow in the dark (about 60 h were required for release of 50% of the label)). Illumination accelerated release. For a high-salt treated sample depleted of PsbP and Q, Ca^{2+} binding was complete between 1 and 2 h, and two sites (K_d = 0.026 and 0.5 mM) were detected. The half time for release was more rapid (25 h) and was accelerated by light. For these samples, steady state assays showed that very little activity was recovered unless Ca^{2+} was also included in the assay buffer. Lastly, removal of all three extrinsic proteins (PsbO, P and Q) produced samples with a drastically impaired ability to bind Ca^{2+}. It should be noted that, these very slow exchange times are sensitive to the presence of a substituting cation; much faster times are observed upon addition of a divalent cation to the medium (Ädelroth et al. 1995 (added Ca^{2+}); Lee and Brudvig 2004 (added Sr^{2+}); Vrettos et al. 2001a (several cations)), in which case exchange is complete in about 4 h. Regardless of the affinity of the OEC for Ca^{2+}, for the time being the stoichiometry seems to be settled. Medium resolution crystal structures predict the presence of on atom of Ca^{2+} for four atoms of Mn in the OEC. This is discussed further below.

Competition between Ca^{2+} and other ions for occupancy of the site in PSII suggest a general trend of effectiveness $M^{3+} > M^{2+} > M^{1+}$ (Vrettos et al. 2001a; van Gorkom and Yocum 2005) with regard to the ability of inhibitory metals to occupy the Ca^{2+} site. The lanthanides that have been tested are all effective at low (50–100 μM) concentrations, and tend to produce inhibitions that are difficult to reverse (Ghanotakis et al. 1985; Bakou et al. 1992). Among divalent metals, Cd^{2+} is the most effective competitive inhibitor (150–300 μM), and there is still no general agreement on the inhibitory potencies of monovalent metals (Na^+, K^+, etc.). For example, Ono et al. (2001) found K^+ to be an effective competitive inhibitor of Ca^{2+} activation of O_2 evolution, and McCarrick and Yocum (2005) and Nagel and Yocum (2005) have shown that K^+ facilitates Ca^{2+} depletion of the OEC. On the other hand, Vrettos et al. (2001a) in their equilibrium binding experiments reported that there is essentially no effect of monovalent metals on Ca^{2+} binding to PSII. Lastly, Ca^{2+} is essential for productive photoactivation of the OEC. This was characterized in detail by Cheniae (see, for example, Chen et al. 1995); the subject has been reviewed recently by Dismukes et al. (2005).

When all the results discussed in this section are taken together, a picture of Ca^{2+} binding to the OEC emerges that is quite complex. It is clear that Ca^{2+} ligation by the OEC is first of all affected by the history of the sample. Use of high ionic strength to release the

PsbP and PsbQ subunits also introduces a lability in the Ca^{2+} binding site. The resulting sample is capable of rapid reconstitution of activity simply by adding the metal to the assay buffer, but the Ca^{2+} site is "open" in the sense that Ca^{2+} is in rapid exchange with the assay medium. Steady state and static measurements of Ca^{2+} binding to these samples produce a wide range of K_d values. Addition of EDTA or EGTA to the buffers used to assay O_2 evolution activity at concentrations sufficient to ligate the metal will inhibit the reconstituted O_2 evolution activity. Acidification of intact PSII to pH 3 for a brief period of time followed by neutralization produces a preparation that retains the extrinsic subunits, and reactivation of the OEC in such a sample requires long incubation times to allow equilibration of Ca^{2+} with its binding site. Whether the extrinsic subunits are present or not, however, Ca^{2+} binding in samples that have not been exposed to high ionic strength occurs with a relatively high affinity. Activity that is retained (after polypeptide extraction) or that can be restored by long-term incubation with Ca^{2+} is by and greatly insensitive to the presence of chelators (Ono and Inoue 1988; Vander Meulen et al. 2002, 2004; Wincencjusz et al., unpublished results; Nagel and Yocum, unpublished results). This would imply that in its native state, even in the absence of PsbP and Q, the Ca^{2+} site in the OEC is either shielded from chelation by structural factors or, alternatively, that the site binds Ca^{2+} with a much higher affinity than do either EDTA or EGTA, whose Ca^{2+} K_d values are about 10^{-11} (Martell and Smith 1974), roughly 7 orders of magnitude higher than the average estimated constant for the OEC Ca^{2+} site ($\sim 10^{-4}$).

Where is Ca^{2+} bound in the OEC?

The recent medium resolution models of PSII structure derived from X-ray diffraction data from *T. elongatus* place Ca^{2+} in close proximity to the Mn cluster. The most recent example is shown in Fig. 1, taken from Loll et al. (2005b). It is now known, that all of the published structures are compromised by radiation damage to the Mn cluster of the OEC. The native higher oxidation states of the Mn atoms of the enzyme in S_1 are reduced to Mn(II), and metal–ligand, and metal–metal distances are drastically modified (Yano et al. 2005). In light of this discovery, even the model shown here has to be viewed as provisional, although the authors have taken steps to minimize the radiation damage to their sample. A recent EXAFS study (which employed lower, non-damaging fluxes of X-rays) on oriented PSII crystals presents a modified version of
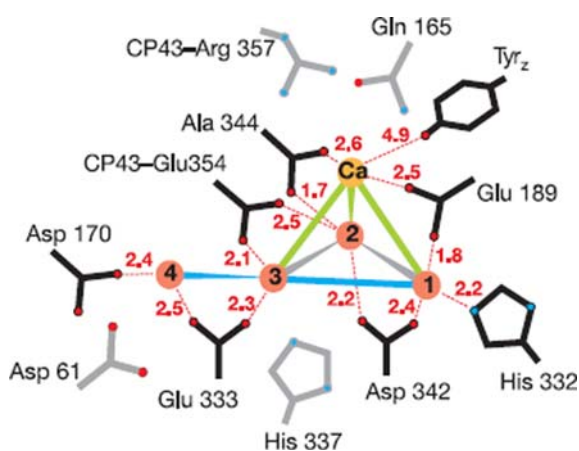
Fig. 1 A model of the Mn_4Ca cluster of the OEC at 3.0 Å resolution (Loll et al. 2005b). The individual Mn atoms are indicated by the numbers 1–4. The numbered residues are from PsbA (D1), with the exception of CP43–Glu354 and Arg 357. Distances (in Å) between cofactors and their ligands are given by the numbers that label the dotted red lines. Reprinted with permission

the relationship between Ca^{2+} and the Mn atoms (Yano et al. 2006). In any case, it is clear that the Ca^{2+} atom in PSII resides near the Mn cluster, at a distance of about 3.4–3.5 Å, and that until the problem of radiation damage is solved, the EXAFS structure is likely to be more reliable in making predictions about the position of Ca^{2+} relative to that of the Mn atoms.

The probable identity of ligands to Ca^{2+} has improved dramatically in a very short time. Ferreira et al. (2004) represented the structure of Ca^{2+} as part of a cubane with three Mn atoms, and no protein ligands to the metal were shown. In contrast, Loll et al. (2005b) proposes the structure of carboxylate oxo anion ligands to Ca^{2+} from Glu189 and Ala 334, both of which provide oxo anion ligands to Mn atoms of the cluster (see Fig. 1), and Yano et al. (2006) add oxo anion ligation from Asp 170. Additional ligands will no doubt be identified as the resolution of the structure improved.

The improving crystal structures have also added information relevant to the recurrent speculations that the extrinsic manganese stabilizing protein (PsbO) may be a Ca^{2+} binding protein. This was suggested originally by Wales et al. (1989). Recently, Kruk et al. (2003) presented evidence that spinach PsbO could bind Ca^{2+} and La^{3+} ions with a K_d of about 10 μM. These authors suggest that cation binding may be essential for proper assembly of the protein into PSII. Heredia and De Las Rivas (2003) used FTIR spectroscopy to probe changes in the structure of PsbO induced by Ca^{2+} binding and found that a small (7–10%) increase in β sheet content could be detected. On the other hand, Loll et al. (2005a) examined the

effects of Ca^{2+} binding on PsbO from *T. elongatus* and concluded that binding of the metal had inconsequential effects on structure. Murray and Barber (2006) analyzed data in Ferreira et al. (2004) and speculate that a Ca^{2+} binding site is present in *T. elongatus* PsbO that is near the lumenal side of PSII. At the same time, all of the currently available models based on crystallographic data (Loll et al. 2005b) or on EXAFS results (Yachandra 2005; Yano et al. 2006) place the functional Ca^{2+} site in the OEC in close proximity to the Mn cluster, and do not predict ligation by amino acid side chain residues of PsbO.

Efforts have been made to probe the location of Ca^{2+} with respect to the structure of PSII and with respect to the chemical reactivity of Mn atoms in the enzyme in the S_1 state. As already described, in spinach PSII preparations that retain all of the extrinsic polypeptides, Ca^{2+} extraction appears to open an access channel to the Mn cluster that can be closed, or partially blocked, by readdition of Ca^{2+} (Vander Meulen et al. 2002, 2004). Extraction of the PsbP and PsbQ subunits exposes the Mn cluster to reduction and loss of Mn(II) catalyzed by hydroquinone and NH_2OH (Ghanotakis et al. 1984c), and it was later shown (Mei and Yocum 1991, 1992) that Ca^{2+} added to PSII in the absence of the PsbP and PsbQ subunits could stabilize the Mn cluster in reduced states. The S_{-1} state formed by hydroquinone reduction was extensively characterized by XAFS and XANES spectroscopy and shown to consist of a 2 Mn(II)/2 Mn(IV) oxidation state (Riggs et al. 1992) that was reversed to the S_1 oxidation state by illumination (Riggs-Gelasco et al. 1996). The hydroquinone reduced samples retained about 80% of their control activities, and it was proposed that Ca^{2+} functioned to stabilize the Mn ligand environment, resulting in the retention of activity even after Mn(II) formation.

When the reactivity of the OEC with reductants was extended to additional reagents (TMPD and dimethylhydroxylamine), it was found that the higher potential reductant (dimethylhydroxylamine; $E^{o'} \sim +0.550$ V) was incapable of facile reduction of the Mn cluster when Ca^{2+} was present (Kuntzleman et al. 2004); a lower potential species (TMPD; $E^{o'} = +0.235$ V) catalyzed reduction of the Mn cluster regardless of whether Ca^{2+} was present or not. On the basis of these observations, it was proposed that Ca^{2+} is positioned topologically so that it blocks access from the external medium to Mn atom or atoms whose redox potential(s) were $\geq +0.550$ V. It can be inferred from these results that some Mn atoms of the cluster must be in a ligand environment where their apparent redox potentials are $\geq +0.235$ V $\leq +0.550$ V. This

would be consistent with the observations (Mei and Yocum 1992) that hydroquinone and NH_2OH react with different populations of Mn atoms in the presence of Ca^{2+}. The higher potential Mn population that is screened from the external medium when Ca^{2+} is present is likely to be the Mn atoms that catalyze water oxidation. The structural data is insufficient at the present time to determine which Mn atoms in the crystallographically-based models might be the metals that catalyze H_2O oxidation.

Ca^{2+} depletion methods revisited

In the section that follows, we discuss the consequences of Ca^{2+} removal for advancement of the S-states of the OEC. The fact that Ca^{2+} depletion appears to block the S-state cycle at the S_2 to S_3 transition might indicate either that S_1 to S_2 transition does not require Ca^{2+}, or that the Ca^{2+} ion is removed only after formation of the S_2 state. The widespread confusion in the literature on this issue, attributed to the variety of methods used to obtain Ca^{2+} depleted PSII, has not yet been resolved. We will try a different presentation here: we first propose our interpretation of the effects of different Ca^{2+} depletion methods and then use that as a framework to discuss the apparently conflicting conclusions in the literature. Removal of the extrinsic PsbP subunit, which also removes PsbQ, is required to allow rapid exchange at the Ca^{2+} binding site. This is not due to a change in Ca^{2+} binding affinity, which is actually increased, but to an increase in exchange rate between the binding site and the medium (Ädelroth et al. 1995). Washing PSII membranes with 1–2 M NaCl in the dark removes PsbP and PsbQ, but does not remove Ca^{2+} from its binding site. On the other hand, illumination during NaCl treatment does result in Ca^{2+} release, due to a much faster dissociation of the metal in the higher S-states (Ädelroth et al. 1995), but the Ca^{2+} is rebound after the treatment, due to a much higher binding affinity in the lower S-states. The inactivation by NaCl-induced Ca^{2+} release is obviously stimulated by illumination (Dekker et al. 1984; Miyao and Murata 1986) and was shown to proceed most effectively in the S_3 state (Boussac and Rutherford 1988a). The possibility of a rapid rebinding of the metal after NaCl treatment in 'Ca^{2+}-free' media, however, seems to have been rejected (Boussac et al. 1990; Kimura and Ono 2001), in spite of warnings that this might occur (Shen et al. 1988; Ono and Inoue 1990b). Nevertheless, the combined observations of (1) a perfectly normal S_1 to S_2 transition on a single turnover, (2) inactivation after the S_3 state has been

formed by two flashes, and (3) a substantial suppression of O_2 evolution in saturating light (Boussac and Rutherford 1988b), clearly suggest that the binding site has now been modified to allow Ca^{2+} binding equilibration in seconds and that the residual Ca^{2+} concentration is enough to out-compete the binding of other species (like Na^+) in S_1 but not in S_3. It is important to note that centrifugation and resuspension in a low-salt medium probably has little effect on the residual free Ca^{2+} concentration, because nearly all Ca^{2+} is non-specifically bound to the PSII preparation. The scheme in Fig. 2A summarizes the effects of this Ca^{2+} depletion procedure.

Calcium chelators like EGTA can prevent the rebinding of Ca^{2+} after NaCl treatment in the light. In this case, the Ca^{2+}-free S_3 state can still decay to S_2, but the Ca^{2+}-free S_2 state is stable for hours, so this treatment leaves most PSII centers trapped in the Ca^{2+}-free S_2 state, which is characterized by a modified EPR multiline signal with more lines and smaller spacings (Boussac et al. 1989; Sivaraja et al. 1989; Ono and Inoue 1990c). On the assumption that Ca^{2+} had been removed from its binding site without the use of a chelator, the modified spectral properties of the S_2 state have been attributed to binding of the chelator to the Ca^{2+}-depleted Mn cluster (Boussac et al. 1990; Kimura and Ono 2001). However, the implication that without chelator the Ca^{2+}-free S_2 state would not be modified seems at odds with the fact that even replacement of Ca^{2+} by Sr^{2+}, which is so similar to Ca^{2+} that it supports O_2 evolution, clearly modifies the EPR (Boussac and Rutherford 1988b) and FTIR (Barry et al. 2005; DeRiso et al. 2006) spectral properties of the S_2 state. Therefore, we prefer the direct interpretation that chelators prevent rebinding of Ca^{2+} after NaCl/light treatment. Since PSII membranes isolated in 'Ca^{2+}-free' media may retain 1000 non-specifically bound Ca^{2+}/PSII, mM concentrations of chelator are required (Stevens and Lukins 2003).

Reconstitution of the extrinsic polypeptides after NaCl/light/EGTA treatment further stabilizes the modified S_2 state, without changing the modified S_2 multiline signal (Boussac et al. 1990). Even with polypeptide reconstitution, the NaCl/light/EGTA treated sample will ultimately (within 2 days, Boussac et al. 1990) decay to the Ca^{2+}-free S_1 state. This corresponds to the situation that can also be obtained in one step by a 5 min exposure of PSII membranes to 10 mM citrate at pH 3 in the dark (Ono and Inoue 1988), which is followed by rebinding of the extrinsic polypeptides upon pH neutralization (Shen and Katoh 1991), see Fig. 2B. The presence of the extrinsic polypeptides in the Ca^{2+}-free S_1 state impedes rapid access
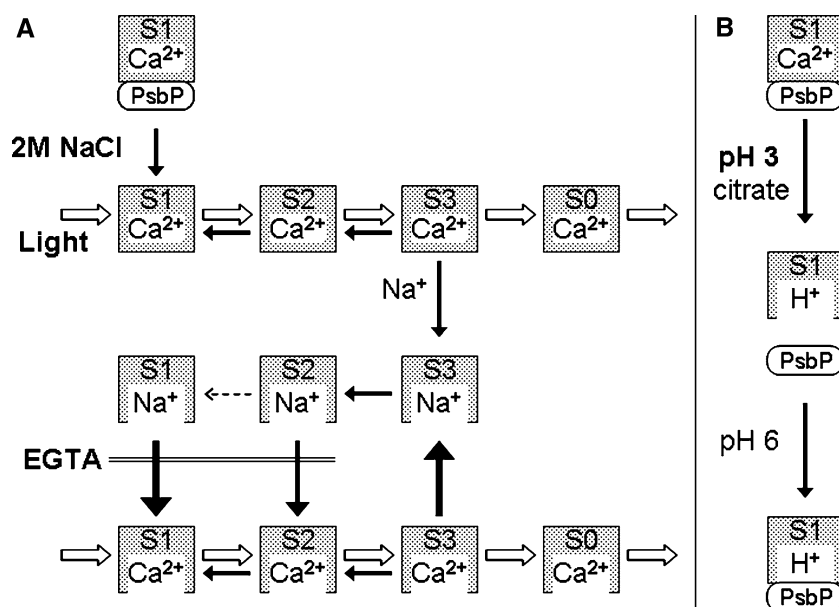
Fig. 2 Ca^{2+} depletion by the salt-washing (**A**) and low pH (**B**) methods. The diagram illustrates this process starting from intact PSII membranes in Ca^{2+} free medium. Both treatments release PsbP and PsbQ from PSII, displace Ca^{2+} from its binding site, and modify the site so that in the case of salt-washing, it exchanges metals rapidly. During exposure to high salt (**A**), this process depends on the Na^+/Ca^{2+} concentration ratio, as well as on the S-state, S_3 having the most rapid exchange rate. Exchange is much slower than the S_3 lifetime, so that prolonged illumination (open arrows) is required to trap all PSII centers in the stable $S_2(Na^+)$ state. If no chelator (for example, EGTA)

is added, subsequent centrifugation and resuspension in low-salt medium decreases the Na^+ concentration much more than the residual Ca^{2+} concentration and may cause rebinding of Ca^{2+} (bottom line). The resulting preparation is then active and shows normal behavior in single-turnover experiments, but its maximum O_2 evolution rate may be much decreased by the accumulation of $S_3(Na^+)$ during illumination, because the Ca^{2+} site remains in fast exchange (seconds, heavy arrows). After low pH treatment (**B**), Ca^{2+} rebinding is avoided by the presence of citrate and by rebinding of PsbP

to the Ca^{2+} binding site and has two additional effects. First, in the absence of Ca^{2+}, PsbP increases the threshold temperature for the S_1 to S_2 transition from 200 K to 250 K (Ono and Inoue 1990a; Ono et al. 1992). Second, after addition of Ca^{2+}, the presence of the extrinsic polypeptides accelerates restoration of the native conformation of the binding site (as evidenced by recovery of EGTA-insensitive O_2 evolution activity), from hours to minutes (Ghanotakis et al. 1984a; Miyao and Murata 1986; Ono and Inoue 1988; Ädelroth et al. 1995).

Why does the OEC contain Ca^{2+}?

The data that are currently available support a structural role for the metal, which is not surprising given the extraordinary number and diversity of proteins in which it plays a central role in conferring structural stability (Kretsinger and Nelson 1976; Lewit-Bentley and Rety 2000; Strynadka and James 1989). The new structural information that's available showing the metal to be linked to Mn by carboxylate bridges is

consistent with a structural function. A structural role alone is, however, incapable of explaining why extraction of the metal blocks S-state advancement. In nearly all cases in biological systems, Ca^{2+} binds H_2O to complete its shell of ligands, and this occasioned proposals (Rutherford 1989; Yocum 1991) that in addition to contributing to the structural stability of the Mn ligand environment of the OEC, Ca^{2+} is a binding site for substrate H_2O molecules that undergo oxidation to produce O_2.

S_3 to S_0 transition

The probable function of Ca^{2+} as a H_2O binding site in the OEC is the centerpiece of contemporary models for the mechanism of water oxidation (Pecoraro et al. 1998; Vrettos et al. 2001b; McEvoy and Brudvig 2004). The major proposition in these mechanisms is that Ca^{2+}, functioning as a Lewis acid, deprotonates H_2O to form Ca^{2+}–OH; the hydroxyl group, an excellent nucleophile, attacks a $Mn^{5+} = O$ group in S_4 to form the O–O bond that precedes reduction of the Mn cluster and release of O_2. The data of Vrettos

et al. (2001a) that examined a number of metals as competing occupants of the Ca^{2+} site fit the proposal in that Ca^{2+} and Sr^{2+} are better Lewis acids than are any of the non-functional metals examined in this study. Hendry and Wydrzynski (2003), studying the exchange rate of bound substrate H_2O molecules, found a 3–5 fold acceleration of the exchange of the most tightly bound H_2O when Ca^{2+} was replaced by Sr^{2+}. This is in agreement with the effect expected if the H_2O is bound to the cation, due to the larger size of Sr^{2+}, and provides further support for the hypothesis that Ca^{2+} is binding this substrate H_2O molecule. Lee and Brudvig (2004) reported that Sr^{2+} substitution also modifies the pH dependence of the maximum rate of O_2 evolution: The lower boundary is up-shifted by one pH unit. The authors propose that this could reflect a pK_a shift of a carboxylate, ligated to the metal, whose unprotonated state is required to accept a proton from the bound H_2O molecule during O–O bond formation:
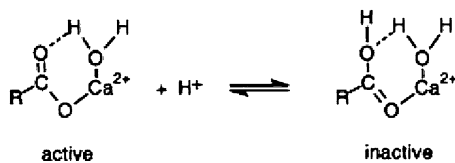
Such an arrangement would indeed imply an essential functional role of the Ca^{2+} ion in the S_3 to S_0 transition that depends critically on its Lewis acidity, as its specificity suggests (Pecoraro et al. 1998; Vrettos et al. 2001a).

S_2 to S_3 transition

Unfortunately, the fact that this attractive hypothesis could account for the Ca^{2+} specificity of the S_3 to S_0 transition seems quite irrelevant, because without Ca^{2+} or Sr^{2+} in the binding site, PSII cannot advance beyond S_2. Depletion of Ca^{2+} or substitution by cations other than Sr^{2+} blocks the S_2 to S_3 transition altogether (Boussac et al. 1989); subsequent illumination only leads to oxidation of Y_Z (Gilchrist et al. 1995). In addition, the effects of Sr^{2+} substitution may not be specific for the S_3 to S_0 transition, because the reduction of $Y_Z\bullet$ on the S_1 to S_2 and S_2 to S_3 transitions is slowed (Westphal et al. 2000). These observations point to a more general role of Ca^{2+}, which might very well be to tune the pK_a of an adjacent amino acid residue that is directly or indirectly required as a proton acceptor that is coupled to oxidation of the Mn cluster, but not specifically during O–O bond formation.

S_1 to S_2 transition

The interpretations of Ca^{2+} depletion procedures presented in the preceding section remove the need to postulate chelator binding to the Mn cluster (Boussac et al. 1990; Kimura and Ono 2001) and the existence of EPR-silent S_2 state(s) (Ono and Inoue, 1989). This clears the way for the simplifying assumption that the production of an unmodified S_2 multiline EPR signal or S_2/S_1 FTIR difference spectrum implies the formation of S_2 in the presence of Ca^{2+}. For the interpretation of EPR measurements, we assume that there is no S_2 state without an accompanying S_2 EPR signal and no Ca^{2+}-free S_2 state unless the S_2 multiline signal is modified. On this basis, we conclude that there is no efficient S_1 to S_2 transition at 0°C when the Ca^{2+} binding site is occupied by K^+, Rb^+, or Cs^+ (Ono et al. 2001). Likewise, we conclude that $S_1(Cd^{2+})$ is not advanced efficiently to S_2 by illumination at 210 K (Ono and Inoue 1989).

For the interpretation of FTIR measurements, if the criticism by Kimura and Ono (2001) is incorrect, this rehabilitates the interpretation of Noguchi et al. (1995), who attributed the characteristic FTIR changes of the S_1 to S_2 transition to a bidentate carboxylate ligand bridging Ca^{2+} and one of the Mn ions that shifts to monodentate Mn ligand in the S_2 state. If this assignment is correct (see Chu et al. 2001), the latest information from X-ray crystallography (Loll et al. 2005b) suggests that the bridging carboxylate could be D1 Glu 189 or Ala 344, but neither of these would agree with the FTIR data (Strickler et al. 2005, 2006). Noguchi et al. (1995) concluded from the overall suppression of the S_1 to S_2 FTIR changes that Ca^{2+} depletion makes this carboxylate dissociate from manganese as well, but since their sample was probably in the $S_1(K^+)$ state (Kimura and Ono 2001), we would conclude that no S_1 to S_2 transition occurred.

For the interpretation of thermoluminescence (TL) measurements, the preceding conclusions would have the following consequences. After Ca^{2+} depletion by the pH 3 method, the normal TL curve showing $S_2Q_A^-$ recombination at about 10°C is shifted to a band near 40°C (Ono and Inoue 1989) that is attributed to $Y_D\bullet^- Q_A^-$ recombination (Demeter et al. 1993; Johnson et al. 1994). Presumably this would indicate that the modified S_2 state has a lower potential than Y_D. However, a similar TL band near 40°C is observed in samples given a single flash in the states $S_1(K^+)$, $S_1(Rb^+)$, and $S_1(Cs^+)$ (Ono et al. 2001). Since there is no EPR evidence for S_2 formation under these conditions, we propose that the upshifted (40°C) TL band is due to recombination of the $S_1Y_Z\bullet Q_A^-$ state. Ono et al.

(2001) rejected the possibility that no S_2 is formed in the absence of Ca^{2+} on the basis of the observation that Ca^{2+} addition after illumination restored the normal $S_2Q_A^-$ TL. However, this might be explained equally well by the conversion of an abnormally stable $Y_Z\bullet$ $S_1(K^+)$ state into $Y_ZS_2(Ca^{2+})$. When Ca^{2+} is replaced by the divalent Cd^{2+}, TL emission occurs at the normal temperature for $S_2Q_A^-$ recombination (Ono and Inoue 1989), although the EPR data of Ono and Inoue (1989) show that S_2 is not formed. A possible explanation for this discrepancy might be that the recombination temperatures of $S_2Q_A^-$ (Ca^{2+}) and $S_1Y_Z\bullet Q_A^-$ (Cd^{2+}) in a TL experiment happen to coincide.

This interpretation leads us to question the proposed origin of the 40°C TL band in the absence of added alkali metal cations, e.g. in pH 3 treated PSII membranes that do show a modified multiline EPR signal after illumination. In these preparations, which contain PsbP, the threshold temperature for the charging of the TL band by a single flash is up-shifted similar to that for Ca^{2+}-free S_2 formation as measured by EPR (Ono et al. 1992), and presumably reflects the flash yield of stable charge separation in S_1. The product measured by TL after a flash at 0°C, above the threshold, might in fact be the Ca^{2+}-free $Y_Z\bullet$ S_1 state, which then converts very slowly and with low yield to the S_2 state that exhibits the modified multiline EPR signal that is observed after illumination for a minute or so. To our knowledge the flash yield of modified multiline formation has not been reported, but flash-induced Mn XANES data (Ono et al. 1993) would suggest an yield of 30–40%, taking into account that the first flash yield may be increased by residual active PSII and that the maximum K-edge shift observed with continuous light is accounted for by the S_1 to S_2 transition alone (Latimer et al. 1998).

There is an additional result that seems to have been overlooked, but which certainly merits inclusion in this discussion. Time-resolved UV absorbance difference measurements on PSII core particles, which lack PsbP and PsbQ and were Ca^{2+}-depleted by the pH 3 method, showed directly that the reaction of $S_1Y_Z\bullet$ to S_2Y_Z was inhibited (Haumann and Junge 1999).

S_0 to S_1 transition, cytochrome b_{559}

There is no information yet on the S_0 to S_1 transition in Ca^{2+}-depleted/substituted PSII. Lockett et al. (1990) proposed that this transition requires Ca^{2+} on the basis of the observation that NaCl treatment at pH 8.3 caused a Ca^{2+}-reversible inhibition of S_2 formation at pH 6.3. However, the apparent conversion of S_1 to S_0 by pH 8.3 treatment (Plijter et al. 1986) was later shown to coincide with the pH at which Cyt b_{559} becomes oxidizable and can compete with S-state advances (Buser et al. 1992), so the samples of Lockett et al. were probably not in the S_0 state but in the S_1 state instead. The connection with cytochrome b_{559} makes it even more intriguing that pH 8.3 appears to facilitate Ca^{2+} depletion by NaCl treatment in the dark. Also, the apparent heterogeneity of the Ca^{2+} affinity might be related to the heterogeneous behavior of cyt b_{559} (in dark-adapted PSII membranes the fraction of low affinity binding corresponds approximately with the fraction of cyt b_{559} present in the reduced state). In view of the proposed role of the cytochrome in a photoprotective electron transfer cycle (Buser et al. 1992), such apparent relations between the Ca^{2+} ion and cyt b_{559} may provide a basis for speculations about a possible involvement of Ca^{2+} in regulating photoprotection.

Y_Z oxidation

Since there appears to be no proof that any of the S-state transitions can occur with reasonable efficiency without Ca^{2+} (or Sr^{2+}) bound to the OEC, one must wonder whether the primary functional role of the metal might be involved with effects on the secondary electron donor Y_Z rather than on the Mn cluster itself. In cyanobacteria, the oxidation of Y_Z by P_{680}^+ is inhibited by Ca^{2+} depletion (Satoh and Katoh 1985; Kashino et al. 1986). Diminished flash yields of Y_Z oxidation have also been reported for Ca^{2+} depleted PSII from higher plants (Boussac et al. 1992) and in lanthanide-substituted preparations, where the effect was shown to disappear at high pH (Bakou and Ghanotakis 1993). Haumann and Junge (1999) studied the behavior of Y_Z oxidation in pea PSII core particles, devoid of PsbP and PsbQ, after Ca^{2+}-depletion by the low pH method. No evidence for S-state advance was observed and Y_Z oxidation had the characteristics of OEC-depleted PSII. Oxidation was slowed by 3 orders magnitude and was dependent on proton release to the medium. It was concluded that the pK_a of the normal proton acceptor, likely D1-His190, was increased from 4.5 to 7. If this is so, then a major role of Ca^{2+} would be to tune the pK_a of His190. Conversely, its unprotonated state would be required for a high affinity of the Ca^{2+} binding site.

Stevens and Lukins (2003) attribute slow Y_Z oxidation to binding of chelators to PSII, but the evidence for that hinges on a comparison of the suppression of O_2 evolution in saturating light by Ca^{2+} depletion to a suppression of nanosecond P^+ reduction in PSII core particles flashed at a repetition rate of 2 Hz, which might differ due to the short life time of the higher S-states in core particles (van Leeuwen et al. 1993).

$Y_Z\bullet$ reduction

A more significant lesion that is induced by Ca^{2+} removal, however, is probably in the reduction of $Y_Z\bullet$ by the Mn cluster. DePaula et al. (1986) were the first to note a correlation between the diminished ability of 200 K illumination to induce the S_2 multiline signal and an increase in the $Y_Z\bullet$ lifetime at room temperature to values normally observed in Mn depleted PSII. Styring et al. (2003) presented evidence suggesting that the block in the reaction $S_2Y_Z\bullet$ to S_3Y_Z is relieved at low pH, with an apparent pK_a of 4.5, although the restored reaction was 3 orders of magnitude slower than in the presence of Ca^{2+}. On the basis of this observation, the main lesion caused by Ca^{2+} removal was attributed to the inability of the OEC to provide the proton required for reduction of $Y_Z\bullet$. This hypothesis was put forward in support of the view that the mechanism of $Y_Z\bullet$ reduction requires proton-coupled electron transfer on every S-state transition (e.g. Hoganson and Babcock 2000). If this is so, then the inhibition of $Y_Z\bullet$ reduction by Ca^{2+} extraction might be due to the loss of a proton that would normally originate from a H_2O molecule bound to Ca^{2+}.

Summary

Even with medium resolution crystal structures of the OEC to serve as guides, the role of Ca^{2+} in H_2O oxidation remains elusive. The data that are currently available can be used to support models in which the metal has both structural and functional roles. Crystallographic, XAS, and biochemical data all place the metal in close proximity to the Mn cluster, where it is required for assembly of the OEC and contributes to the stability of Mn ligation. Proximity to the Mn cluster is also central to models for Ca^{2+} function in the mechanism of O_2 evolution. In this case, the ability of Ca^{2+} to function as a Lewis acid provides the underpinning for reasonable models for the mechanism by which both Mn and Ca^{2+} function to catalyze the formation of the first O–O bond in PSII. At the same time, the probability that most or all S-state transitions require Ca^{2+} suggests that current models for its role as a catalytic component of the OEC may need to be expanded and/or modified to include additional contributions of Ca^{2+} to the mechanisms of Mn and H_2O oxidation and/or $Y_Z^\bullet$ reduction. Likewise, the complexities of interactions between Ca^{2+}, the intrinsic polypeptides of PSII, and the extrinsic subunits deserve further characterization regardless of the insights provided by present and future models derived from crystallographic data. For example, information on the extent to which binding of extrinsic subunits to the intrinsic core of PSII affects Ca^{2+} binding to its site in the OEC would be most useful. As the resolution of PSII crystal structures improves, it should be possible to begin to identify the pathways by which H_2O enters the OEC and O_2 exits this site. This will provide new opportunities to probe the role of Ca^{2+} in PSII as both a catalytic and structural component of this important enzyme system.

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