The functional role of calcium in photosystem II

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The functional role of Ca^{2+} in Photosystem II

Mohamed Miqyass

The functional role of Ca^{2+} in Photosystem II

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

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door

Mohamed Miqyass

Geboren te Ouled si Lahcen, Marokko, in 1978

Promotiecommissie

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	Dr. J. P. Dekker (Vrije Universiteit Amsterdam)
	Dr. Alia
	Prof. Dr. J. M. van Ruitenbeek

Dis : "En vérité, ma Salat, mes actes de dévotion, ma vie et ma mort appartiennent à Dieu, Seigneur de l'Univers. A Lui nul associé ! Et voilà ce qu'il m'a été ordonné, et je suis le premier à me soumettre."

Pour Selma

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1 Introduction

1.1 PHOTOSYNTHESIS, ENERGY AND REDUCING POWER

The energy that keeps life on earth going is provided by sunlight. Photosynthetic organisms capture that energy by absorbing light in their 'light-harvesting complexes'. These are proteins that contain chlorophylls and/or accessory pigments such as carotenoids or phycobilins, whose excitation energy is transferred to chlorophylls. Most of the chlorophylls serve merely as an antenna: they absorb light and transfer the excited state to neighboring chlorophyll molecules. A tiny fraction, in plants about 1 in 200, of the chlorophylls is 'photochemically active'. They initiate a chain of electron transfer reactions when they are excited electronically by absorption of a photon or by exciton transfer from the antenna chlorophylls. They are rigidly positioned in 'reaction center' proteins, together with the redox-active cofactors that mediate these reactions, such that almost every excitation leads to a stable charge separation. The reaction centers, like most light-harvesting complexes, are embedded in a membrane and the charge separation results in the transfer of an electron from one side of the membrane to the other. Due to charge compensation by H^+ release on the oxidizing side and H^+ binding on the reducing side, the charge separation in turn causes a H^+ concentration difference (ΔpH) between the two sides of the membrane, which provides the driving force for the phosphorylation of ADP to ATP, adenosine triphosphate, the universal 'energy currency' of biochemistry.

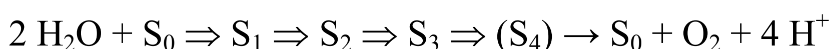
In some organisms, notably the photosynthetic purple bacteria, this may be the main result of photosynthesis. A protonated reductant, ubiquinol, diffuses from its site of formation at the reducing side to the other side of the membrane where it is oxidized, via cytochrome c and the cytochrome b/c complex, by the same reaction centers, so that a cyclic eltron transfer path results. In most photosynthetic organisms, however, photosynthetic electron transport is mainly non-cyclic and produces, in addition to ATP, reducing power in the form of reduced cofactors like NADPH (nicotinamide adenine dinucleotide phosphate). Reducing power is required for the synthesis of organic matter, 'biomass', because the available inorganic sources of its constituents, like carbon dioxide, nitrate, and sulfate, are highly oxidized. The ultimate electron source that photosynthesis uses on a global scale for this purpose is water. This type of photosynthesis is called oxygenic, because the oxidation of water produces oxygen, O_2, as a waste product.

In oxygenic photosynthesis, which is carried out by cyanobacteria and by the chloroplasts of algae and higher plants, two types of photosystem operate in series. Charge separations in the reaction center of Photosystem II (PSII) reduce plastoquinone to plastoquinol and generate a sufficiently strong oxidant to use water as electron source. Plastoquinol is oxidized by the cytochrome b6-f complex, which transfers electrons to the small copper protein plastocyanin. Charge separations in the reaction center of Photosystem I (PSI) generate the required reducing power, using plastocyanin as the electron donor. In the chloroplasts of higher plants the two photosystems are largely segregated in different domains of the photosynthetic membrane, which locally forms stacks of tightly appressed membrane discs, the so-called grana, where most of PSII is located. By selective solubilization of the more exposed regions of the membrane system with a detergent, it is possible to isolate such membrane stacks containing almost exclusively PSII (Berthold et al. 1981) that have been used for all experiments described in this thesis. In these PSII preparations the oxidizing side of the reaction center is exposed to the medium, but retains its protective shield of extrinsic polypeptides and is capable of sustained O_2 evolution on illumination in the presence of an artificial electron acceptor.

For a general introduction to photosynthesis, see Blankenship (2002). All aspects of PSII are covered in a recent multi-author book (Wydrzynski and Satoh 2005).

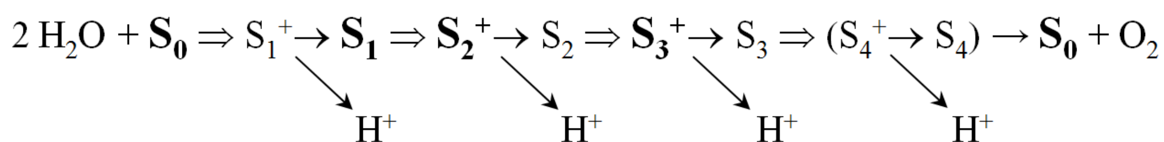
1.2 WATER OXIDATION, A 4-ELECTRON REACTION

Photochemical charge separation is a one-electron event, producing the oxidized PSII reaction center chlorophyll P_{680}^{+}, which in turn oxidizes the secondary electron donor Y_Z, a tyrosine residue of the reaction center protein called PsbA, or D1. The oxidation of water to oxygen is energetically feasible only as a concerted 4-electron event, or perhaps two 2-electron events, and the release of highly reactive intermediates like $OH^{\bullet}$, H_2O_2, or $O_2^{\bullet -}$, would destroy reaction center proteins (Krishtalik 1986). Intact PSII releases an O_2 molecule once every 4 photoreactions. This is strikingly demonstrated by O_2 measurements on illumination of dark-adapted PSII by a series of single-turnover flashes, i.e. flashes that are strong enough to cause charge separation in every PSII and short enough to allow only a single charge separation in each PSII. The O_2 yield shows a periodicity of 4 with maxima on flash numbers 3, 7, 11, etc. (Joliot et al. 1969), indicating that PSII accumulates the 4 oxidizing equivalents before O_2 is released and the first of these is stable in the dark (Kok et al. 1970):



$$2\,H_2O + S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow (S_4) \rightarrow S_0 + O_2 + 4\,H^+$$

Although each oxidation in this 4-step 'S-state cycle' is initiated by $Y_Z\bullet$, they differ in properties like kinetics, temperature dependence and pH dependence. Many of these differences can be rationalized by the need to restore electroneutrality by H^+ release after each oxidation, in order to make the next oxidation thermodynamically feasible (Dau and Haumann 2007). The substrate water molecules may not be deprotonated until the last step, as indicated in the scheme above, but the measured sequence of H^+ release is actually 1, 0, 1, 2 for the $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow S_0$ cycle. Consequently, the net charge of the system is increased by one in the S_2 and S_3 states, compared to the S_0 and S_1 states. Taking these considerations into account and showing the classical S-states in boldface, the scheme becomes:



Unlike the oxidation of S_0, the oxidation of S_1 is not followed by spontaneous H^+ release. As a consequence, electron transfer to Y_Z• on the subsequent flash will be rate-limited by the H^+ release, because the redox potential of S_3^{2+}/S_2^+ is prohibitively high. The oxidation of S_3^+ also must await H^+ release, but here electron transfer to Y_Z• is rate-limited by the 2 ms rate constant of water oxidation, presumably because the redox potential of S_4^+/S_3 is somewhat higher than that of Y_Z•/Y_Z so that S_4^+ or S_4 , if such a state exists, does not accumulate as a detectable intermediate. The actual mechanism of water oxidation and O–O bond formation remains to be elucidated (see Hillier and Messinger 2005 for a summary at least 14 different models that have been proposed). Even as a concerted 4-electron event, the reaction can probably only occur because it is coupled energetically to a simultaneous binding of the protons to sufficiently strong bases, which might be the same sites that were deprotonated during the cycle and would be strong bases in the S_0 state (Krishtalik 1986).

The distinctive periodicity of 4 with flash number has facilitated the discovery of numerous spectroscopic features that accompany one or more of the oxidation states and have provided a wealth of information on the O_2-evolving complex (OEC) (Debus 1992; Nugent 2001; Wydrzynski et al. 2005). The successive oxidation states are associated with a cluster of 4 oxo-bridged Mn atoms and 1 Ca^{2+} ion bound to the PSII reaction center protein. In addition, a Cl^- ion is essential for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions (Wincencjusz et al. 1997). Its role may be to tune the potential of one of the Mn atoms by preventing stabilization of its Mn(IV) state by OH^- (van Gorkom and Yocum 2005). The Ca^{2+} ion is not only an essential structural component. Current models propose a specific role for Ca^{2+} in the final step, the formation of the O–O bond. This may be so, but there seems to be no evidence that Ca^{2+} is required for the $S_3 \rightarrow S_0$ transition. It is required for the $S_2 \rightarrow S_3$ transition. There is no general agreement on its possible requirement for the $S_1 \rightarrow S_2$ transition, which is of special interest if this transition does not involve proton transfer.

1.3 THIS WORK

The research described in this thesis was initiated to determine the Ca^{2+} dependence of the successive S-state transitions by UV absorbance difference spectroscopy. The approach used here would be analogous to that used successfully by Wincencjusz et al. (1997) to demonstrate that Cl^{-} is required for S-state advance from S_2 and from S_3 only. First, the conditions would be established to obtain a PSII preparation that shows a clear dependence of its S-state absorbance changes on the addition of Ca^{2+}. Then methods would be developed to insert the Ca^{2+} in, or remove it from, its functional binding site so quickly that this can be done between flashes in a series, well within the lifetime of the higher S-states.

In spite of much effort, however, known methods of Ca^{2+} depletion seemed to fail, unless the extrinsic polypeptides were bound to PSII and prevented rapid exchange at the Ca^{2+} binding site. Finally it was concluded that the literature on this subject had to be re-examined in detail. As a result, this thesis begins with an extensive literature survey, Chapter 2, which proposes a different interpretation and examines the consequences of that interpretation for the conclusions from a wide variety of published investigations.

In the following chapters experimental evidence is presented that supports the literature interpretations proposed in Chapter 2 and provides new information on the properties of the Ca^{2+} binding site that leads to a different view of the role of Ca^{2+} in photosynthetic O_2 evolution. Chapter 3 introduces the methods used and shows the results obtained after conventional methods of Ca^{2+} depletion. Chapter 4 describes the surprisingly effective Ca^{2+} depletion by exposure to a high K^{+} concentration and shows that this inactivates the $S_1 \rightarrow S_2$ transition. Chapter 5 finally does provide the initially sought information on the S-state dependence of the Ca^{2+} binding characteristics and effects of Ca^{2+} replacement by other metal ions in each S-state.

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2 The PSII Ca^{2+} site revisited*

*M. Miqyass, H.J. van Gorkom and C.F. Yocum (2007)
Photosynth. Res. 92: 275-287

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[1]. 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, H.J. and Yocum, C.F. in Wydrzynski TJ and 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+}.

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[1] **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.

2.1 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+} (Kd = $\sim 10^{-6} - 10^{-7}$ 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 tell, 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 a somewhat 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 PsbQ (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).

2.2 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 QA– 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 S3 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 sec), 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 ^oC) reconstitutions of the Ca^{2+} site proceeded more rapidly than at 4 ^oC, 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 ^oC 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). Mention should also be made here of 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 to binding to the OEC Ca^{2+} site or to Mn binding sites in the 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 hrs 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 hrs 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 one and two hrs, and two sites (K_d =0.026 and 0.5 mM) were detected. The half time for release was more rapid (25 hr) 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 four hr. 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) find 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 report 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 of 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 large 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 (~10^{-4}).

2.3 WHERE IS CALCIUM 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 Figure 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

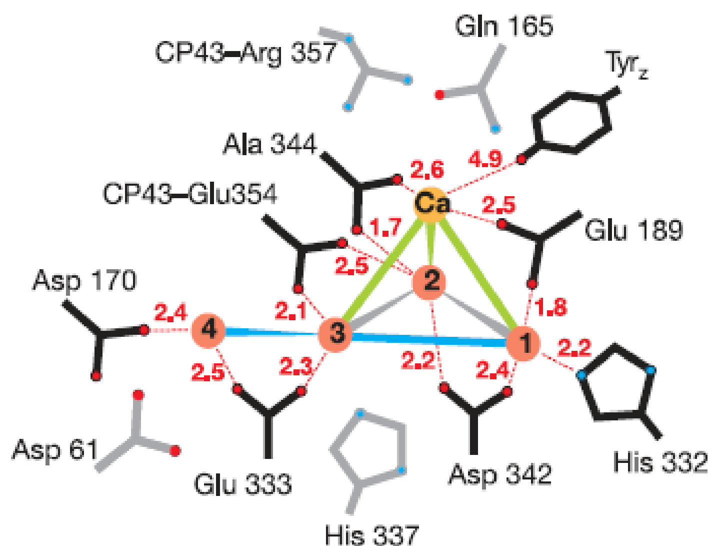


Figure 1. A model of the Mn_4Ca cluster of the OEC at 3.0 Å resolution (Loll et al. 2005b). The individual Mn atoms are indicated by the numbers 1-4. The numbered residues are from PsbA (D1), with the exception of CP43-Glu354 and Arg 357. Distances (in Å) between cofactors and their ligands are given by the numbers that label the dotted red lines. Reprinted with permission.

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 the relationship between Ca^{2+} and the Mn atoms (Yano et al. 2006). In any case, it's 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. The structure of Ferreira et al. (2004) represented Ca^{2+} as part of a cubane with three Mn atoms, and no protein ligands to the metal were shown. In contrast, the structure of Loll et al. (2005b) proposes carboxylate oxo anion ligands to Ca^{2+} from Glu189 and Ala 334, both of which also 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 improves.

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 Mn(II) loss 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 a 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. There are insufficient structural data at the

present time to determine which Mn atoms in the crystallographically-based models might be the metals that catalyze H_2O oxidation.

2.4 CALCIUM 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 somewhat 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 t hat the residual

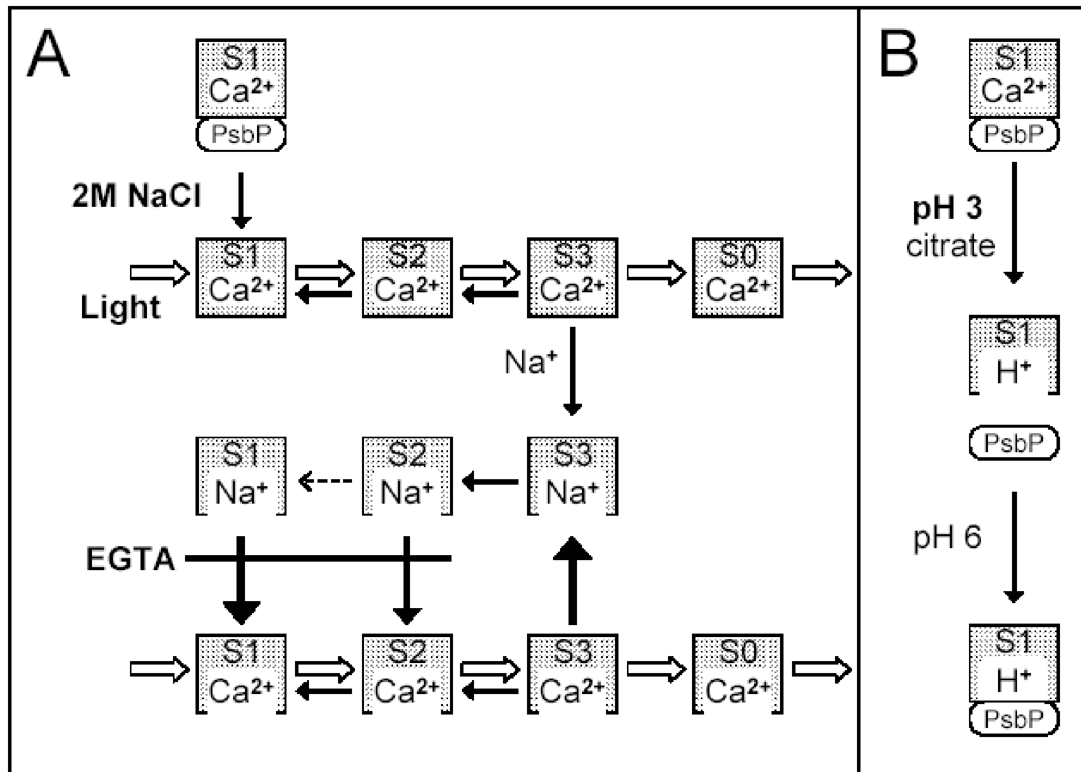


Figure 2. Ca^{2+} depletion by the salt-wash (A) and low pH (B) methods. The diagram illustrates this process starting from PSII membranes in Ca^{2+} free medium. Both treatments release PsbP and PsbQ from PSII, displace Ca^{2+} from its binding site, and modify the site so that in the case of salt-washing, it exchanges metals rapidly. During salt-treatment (A), this process depends on the Na^{+}/Ca^{2+} concentration ratio as well as on the S-state, S_3 having 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 O2 evolution rate may be much decreased by the accumulation of S3(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.

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 straightforward 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 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).

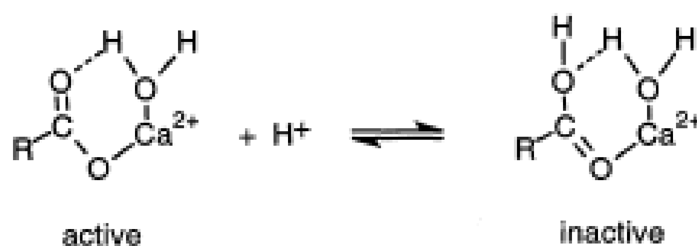
2.5 WHY DOES THE OEC CONTAIN CALCIUM?

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.

2.5.1 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 somewhat 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].

2.5.2 *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.

2.5.3 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 S2 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 also 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 a 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).

2.5.4 S_0 to S_1 transition, *cytochrome* b559

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. probably were 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.

2.5.5 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).

2.5.6 $Y_Z^{\bullet}$ 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_2 $Y_Z^{\bullet}$ 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+}.

2.6 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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3 Comparison of Ca^{2+} depletion methods by S-state absorbance changes in a flash series

ABSTRACT

The inactivation of photosystem II membranes by Ca^{2+} depletion was investigated by measurement of the UV absorbance changes associated with the S-state cycle of the oxygen evolving complex on illumination by a series of saturating flashes. Inactivation by low pH treatment was readily confirmed and was obvious already on the first flash. Subsequent removal of the extrinsic polypeptides PsbP and PsbQ led to reactivation by residual Ca^{2+} associated with the PSII membranes. Calcium depletion by NaCl treatment appeared to inhibit the S-state cycle during exposure to the high NaCl concentration, but not on the first flash, and the inhibition disappeared after resuspension in low salt medium. An inhibition equivalent to that induced by low pH treatment could be obtained, but only by rigorous exclusion of residual Ca^{2+}. The results support the proposal in the previous chapter that rebinding of the extrinsic polypeptides or the presence of a chelator after NaCl treatment is essential to avoid reactivation of PSII by rebinding of residual Ca^{2+} to samples that have been depleted of the metal. The steady state inhibition of O_2 evolution in such samples is most likely due to rapid Ca^{2+} loss during measurement, and not to its depletion from samples by the high salt treatment.

3.1 INTRODUCTION

The re-interpretation of the effects of Ca^{2+} depletion by the 'salt-wash procedure' that was proposed in the previous chapter (Miqyass et al 2007) may account for some inconsistencies and assumptions in the literature. This assertion requires experimental verification, and it also raises new questions that require an approach that differs from those that have previously been applied to this problem. Earlier published results were obtained using methods that characterized a Ca^{2+} depleted state after its formation. In most instances this involved continuous illumination and/or cryogenic temperatures and characterization of the resulting state e.g. by EPR or thermoluminescence. These techniques are not well-suited for analysis of the reactions that produced such a state. Resolving the S-state dependence of Ca^{2+}-dependent reactions presents a substantial challenge, because it was shown early on that > 100 flashes or many minutes of continuous illumination were required for thorough Ca^{2+} depletion (Dekker et al. 1984a; Miyao and Murata 1986). On the basis of these observations, one would expect very little effect on Ca^{2+} retention by PSII samples exposed to a few single turnover flashes. Nevertheless, Boussac and Rutherford (1988) reported a significant dependence of the inactivation by NaCl treatment on the number of pre-illuminating single-turnover flashes, suggesting that the process was significantly more effective after 2 saturating flashes than in the dark-adapted state. If so, it should be possible to monitor the effects of Ca^{2+} release in real time and study its S-state dependence with a suitable technique.

A convenient technique for monitoring S-state transitions is measurement of the flash-induced UV absorbance changes associated with these transitions (Pulles et al. 1976). This provides a direct and quantitative probe that can be measured at physiological temperatures (Dekker et al. 1984c; van Leeuwen et al. 1993) and, if needed, within seconds after additions to a flashed sample have been made (Wincencjusz et al. 1997). So far the only study of Ca^{2+} depleted PSII employing this technique was that by Haumann and Junge (1999) on the retardation of electron transfer from the secondary donor, tyrosine Y_Z, to the oxidized reaction center chlorophyll P_{680}^{+}. No evidence was found for electron transfer from the O_2 evolving manganese cluster to Y_Z• after a flash, even though the sample was Ca^{2+} depleted in darkness by the pH

3 / citrate method of Ono and Inoue (1988) and should therefore be in the S_1 state. It is not clear if the absence of an S_1 to S_2 transition in this case can be attributed to the use of PSII core particles depleted of the PsbP and PsbQ extrinsic polypeptides, rather than the usual PSII membranes.

An investigation of the mechanisms and effects of Ca^{2+} depletion of PSII by UV absorbance difference spectroscopy was initiated with a view towards clarifying some of the contradictory data in the literature. Here we describe how the UV absorbance difference measurements were carried out and compare the results obtained from samples treated by the two commonly used Ca^{2+} depletion methods, exposure to either high ionic strength, or to low pH. The results reported here show that suppression of S-state absorbance changes is readily observed after treatment of PSII with low pH. Exposure to high ionic strength produces samples that retain period-4 oscillations, and additional measures are needed to produce Ca^{2+} depletion.

3.2 MATERIALS AND METHODS

Spinach PS II membranes were isolated according to Berthold et al (1981) with modifications (Ghanotakis et al. 1984b) and stored in 0.4 M sucrose, 50 mM MES and 10 mM NaCl, pH 6.0. All subsequent operations were carried out at 4 ^{o}C in darkness unless otherwise noted. For Ca^{2+} depletion by low pH treatment (Ono and Inoue 1988), PSII membranes were suspended in 0.4 M sucrose, 50 mM MES pH 6.0, centrifuged 20 min at 48 000 x g and resuspended at 0.5 mg Chl /ml in the same buffer. An equal volume of 0.2 M citrate, 0.4 M sucrose, 10 mM NaCl, pH 3.0 was added. After a 5 min incubation the pH 3 treatment was stopped by adding 8 volumes of 0.4 M sucrose, 50 mM MES pH 6.0, 30 mM betaine and 30 mM tetramethylammonium chloride (TMACl) (buffer A.); the TMACl was used to provide Cl^{-} without adding a metal cation that could interfere with the Ca^{2+} site; betaine was used to prevent aggregation. After mixing for 5 minutes, the pH-treated PSII membranes were centrifuged and resuspended in buffer A.

For Ca^{2+} depletion by salt-washing (Ghanotakis et al. 1984a; Miyao and Murata 1984), PSII membranes were suspended at 0.4 mg Chl/ml in buffer A. After stirring for 5 min, an equal volume of 4 M NaCl and 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was added,

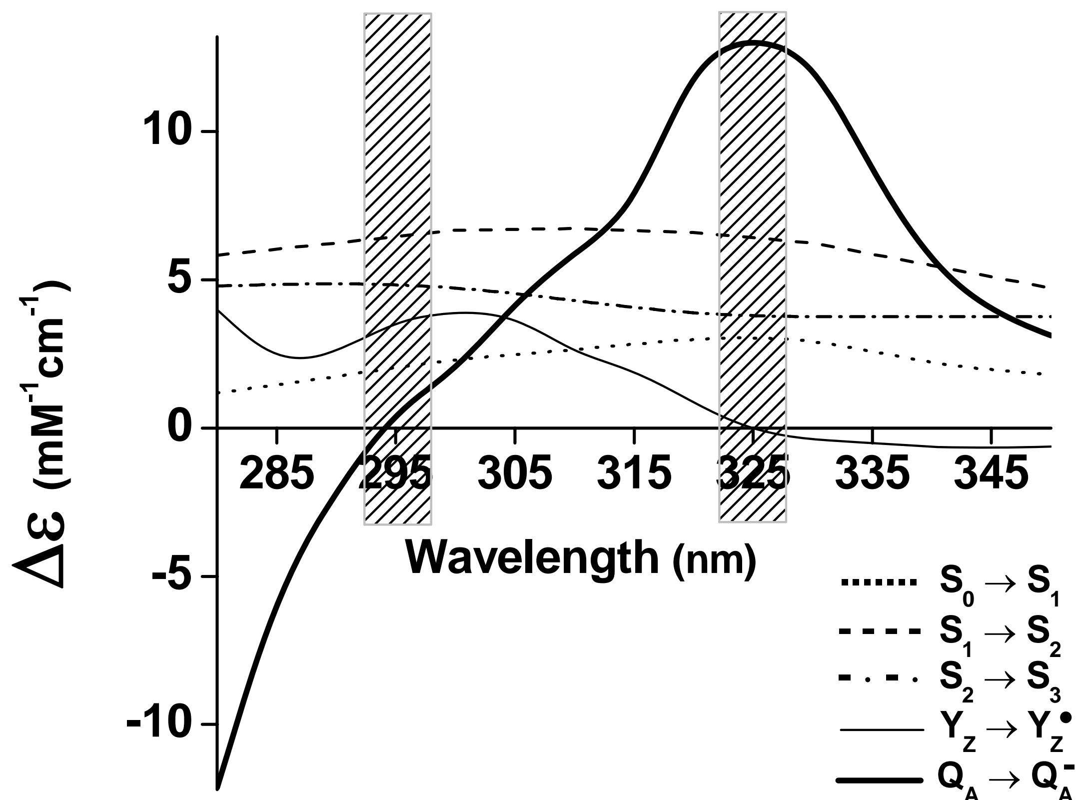

Figure. 1. Absorbance difference spectra of S-state transitions, Y_Z oxidation, and Q_A reduction. Grey bars show the half width of the spectral profile of the measuring light used here, which was centered either at 295 or at 325 nm to minimize Q_A or Y_Z contributions, respectively. Based on data from van Leeuwen et al. (1993) and Dekker et al. (1984b).

giving final concentrations of 0.2 mg Chl/ml, 2 M NaCl, and 1 mM EGTA. The mixture was incubated for 30 min in the dark and then exposed to room light for 30 min. To assure homogeneous light exposure, the sample was spread in a thin layer on the flat bottom of a large Erlenmeyer flask. The NaCl-washed PSII membranes were centrifuged for 20 min at 48 000 x g and resuspended in buffer A.

Flash-induced S-state transitions were measured by UV absorbance changes with a single beam absorbance difference spectrophotometer. As shown by van Leeuwen et al. (1993), the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, and $S_2 \rightarrow S_3$ transitions cause a small absorption increase throughout the near ultraviolet (see Figure 1), which is reversed on the $S_3 \rightarrow S_0$ transition. The changes are most conveniently measured near 295 nm, where interfering absorbance changes due to the concomitant reduction of electron acceptors are small. There is a significant contribution at 295 nm from the transient oxidation of the

secondary electron donor, tyrosine Z ($Y_Z \rightarrow Y_Z\bullet$) (thin line). This absorbance change is normally short-lived and seen mainly during the 2 ms $S_3 \rightarrow S_0$ transition, but this will change when oxidation of the manganese cluster is inhibited by Ca^{2+} depletion. When $Y_Z\bullet$ reduction is slower than the flash repetition rate, it accumulates and the photooxidized primary donor P_{680}^+ back reacts with Q_A^- in 0.3 ms (Dekker et al. 1984a). This reaction contributes only slightly at 295 nm (Gerken et al. 1989) and is detected most easily by the decay of Q_A^- absorption at its 325 nm peak (Figure 1, heavy line). Absorbance changes were therefore detected at both 295 and 325 nm, with a 0.1 ms time resolution. The number of measurements averaged for an acceptable signal to noise ratio was minimized (< 20) by using wide monochromator slit widths (grey bars in Fig. 1) and high current through the halogen lamp. The actinic effect of the measuring light was minimized by blocking it between flashes with a fast mechanical shutter. Saturating actinic flashes from a YAG laser (532 nm, 6 ns FWHM) were used at a frequency of 4 Hz. A small electronic artifact was removed from the signal; the first point used was at 0.2 ms after the flash (the $t_{1/2}$ of P_{680}^+ Q_A^- recombination in Ca^{2+}-depleted PSII). Fits to the Kok model were obtained in Matlab by decomposition of the flash-induced kinetics into a sum of exponentials with flash number and wavelength independent time constants, followed by optimization of the model parameters to fit the amplitudes of those components using the extinction coefficients of Figure 1.

3.3 RESULTS

Figure 2 shows the absorbance changes at 295 nm and 325 nm induced by a series of single-turnover flashes in a preparation of intact PSII membrane fragments, measured as described in Materials and Methods. The flashes were spaced at 250 ms and absorbance was recorded from 20 ms before to 50 ms after each flash. The changes at 295 nm are composed of a stable component due to the absorbance difference between the successive S-states, and a transient with a time constant of 2 ms caused by the reduction of $Y_Z\bullet S_3$ to $Y_Z S_0$ that was shown to be proportional to the amount of oxygen produced (van Leeuwen et al. 1990). The amplitudes of these changes show a characteristic damped oscillation with flash number that can be described quantitatively by the Kok model for the S-state cycle (Kok et al. 1970).

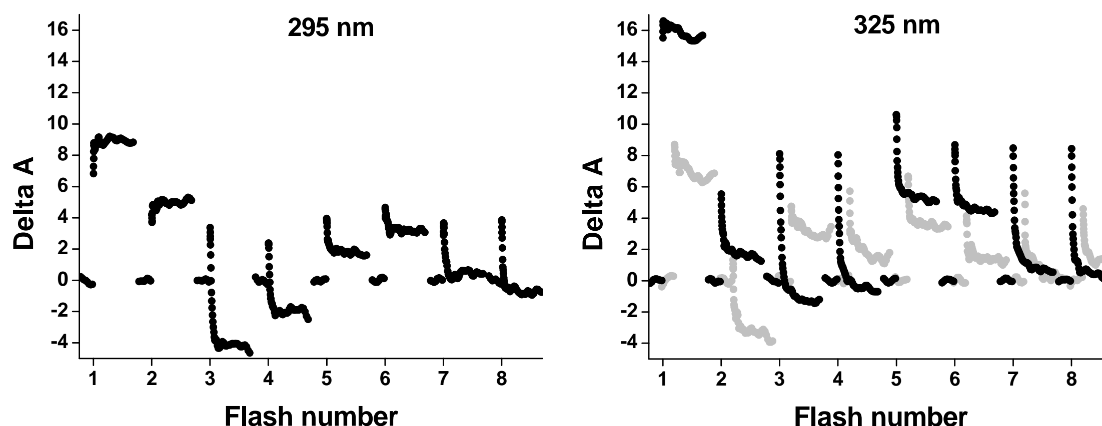

Figure. 2. Flash-induced absorbance changes in PSII membranes measured at 295 nm (left) and 325 nm (right), measured from 20 ms before to 50 ms after each flash. Grey traces (at small x-offset for clarity): 325 nm minus 295 nm. Concentrations: 200 µg Chl/ml in buffer A with 100 µM DCBQ and 400 µM ferricyanide. ΔA absorbance units (250 mM Chl)$^{-1}$cm^{-1}, or 10^4 times the measured value, flattening-corrected, corresponding to Δεunits in Fig. 1.

At 325 nm, the S-state cycle contributes similar, though not identical, absorbance changes, superimposed on absorbance changes due to semiquinones produced by the one-electron reduction of Q_A, Q_B, or the quinone added as an artificial electron acceptor, DCBQ (Figure 1). By subtracting the changes at 295 nm from those at 325 nm, a pattern is obtained that is dominated by the semiquinone kinetics. Figure 2 reveals their multiphasic decay and initial period 2 oscillation, in spite of the presence of 0.1 mM DCBQ and 0.4 mM ferricyanide as artificial electron acceptors. These complications, and also the fact that the first flash causes a larger increase at 295 nm than can be explained by the S-state cycle have been discussed by Dekker et al (1984c).

When PSII membranes were Ca^{2+} depleted by a 5 min exposure to pH 3 / citrate according to Ono and Inoue (1988), the pattern of oscillating flash-induced absorbance changes at 295 nm was quite similar to that of an untreated PSII sample, but the amplitudes of the absorbance changes were reduced 4-5 fold (Figure 3, black symbols). Residual activity must arise from PSII centers that retain Ca^{2+}, while Ca^{2+} depleted centers contribute very little to the signal except for some increase on the first flash. This increase is too small to be due to an $S_1 \rightarrow S_2$ transition, because even when all manganese oxidation is prevented, e.g. by Tris treatment, the amplitude on the first flash

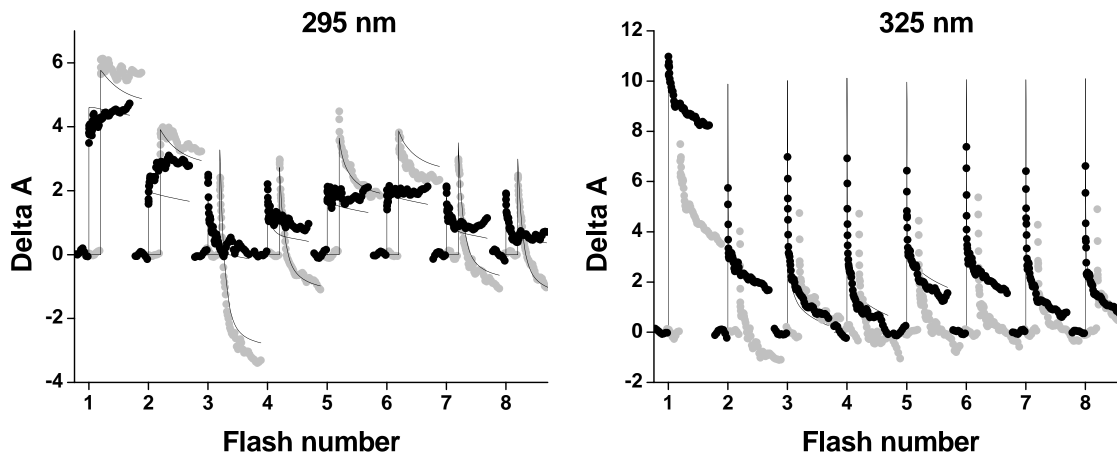

Figure. 3. PSII membranes Ca^{2+} depleted by 5 min pH 3 / citrate treatment, measured as in Fig. 2 at 295 nm (left) and 325 nm (right, 325 minus 295 nm in grey). Grey traces in left frame: PsbP and PsbQ removed by 15 min pH 7.5 / 50 mM sulfate wash after the low pH treatment, using chelex treated buffers. Lines: fit to the Kok model, indicating that 83 % of the PSII centers could only oxidize Y_Z after pH 3 treatment, but only 50 % were still inhibited after removal of PsbP and PsbQ.

due to Y_Z oxidation is still 2.5-3 on this scale (Dekker et al. 1984b). At 325 nm the absorption increase after the first flash indicates formation of a long-lived semiquinone in the majority of PSII centers, whereas from the second flash on most of the flash-induced increase decayed with a time constant of 0.3 ms.

This is consistent with the interpretation that the first flash produces a stable charge separation that generates $Y_Z^{\bullet}$; on subsequent flashes $P_{680}^{+}Q_A^{-}$ recombination occurs.

The results shown in Fig. 3 document the inhibition of the $S_1 \rightarrow S_2$ transition after pH 3 / citrate treatment, confirm the report of Haumann and Junge (1999) and show that this inhibition is not due to use of PSII core particles lacking the extrinsic PsbP and PsbQ subunits. The presence of these polypeptides in the pH 3 preparation used here is documented by the fact that reactivation by added $CaCl_2$ was very slow (~1 h). Removal of PsbP and PsbQ by pH 7.5 / sulfate treatment (Wincencjusz et al. 1997) largely restored the absorbance changes of the S-state cycle, even when thoroughly chelex-treated calcium free buffers were used (grey traces in Fig. 3, left). This reactivation was, in turn, suppressed by EGTA (half inhibition at 100 μM, not shown). Apparently the amount of Ca^{2+} introduced by the PSII membranes

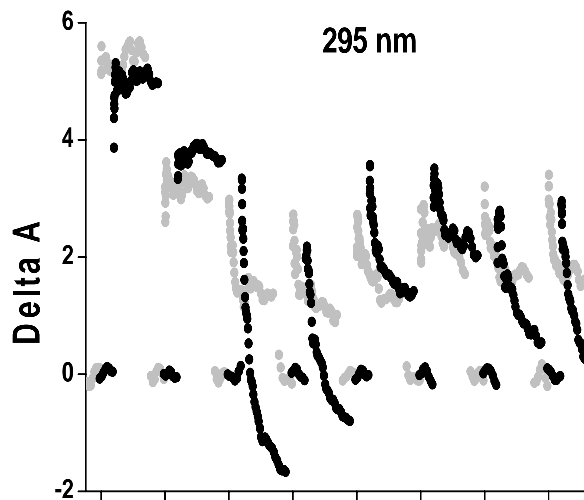

Figure. 4. PSII membranes Ca^{2+} depleted by exposure to 2 M NaCl / 1 mM EGTA for 1 hour (30 min in dark, 30 min in room light). Grey traces: during incubation, measured in the presence of 2 M NaCl / 1 mM EGTA. Black: after resuspension in buffer A.

themselves and not washed out with the citrate was > 100 µM and sufficient to restore activity, but had no access to the functional binding site as long as the extrinsic polypeptides were in place. The lines in Fig. 3 show a global fit to the Kok model allowing a fraction of the PSII centers to be unable to perform any S-state advance, instead producing a long-lived Y_Z•, or $P_{680}{}^{+}Q_A{}^{-}$ recombination if Y_Z• was still present. This fraction was 83 % after pH 3 treatment and 50 % after removing PsbP and PsbQ, while the long-lived Y_Z• showed 32 % decay between flashes.

Figure 4 shows results obtained after Ca^{2+} depletion by salt-washing. The usual treatment with 1-2 M NaCl to remove PsbP and PsbQ - which originally led to the discovery of the Ca^{2+} requirement for oxygen evolution - did not seem to suppress the oscillating absorbance change in a flash series. However, when a measurement after 30 min incubation in 2 M NaCl / 1 mM EGTA was carried out in that mixture, a strong suppression of the S-state cycle was observed after the first two flashes (Fig. 4, grey traces). The result obtained after centrifugation and resuspension in low salt medium was variable and seemed to depend critically on the residual Ca^{2+} contamination. Unless the suspending buffer medium had been rigorously chelexed the absorbance oscillation was fully restored without Ca^{2+} addition. These results differ from the untreated control only in an increased miss probability and a much slower S_3 to S_0 transition (9 ms); these effects result from the high ionic strength

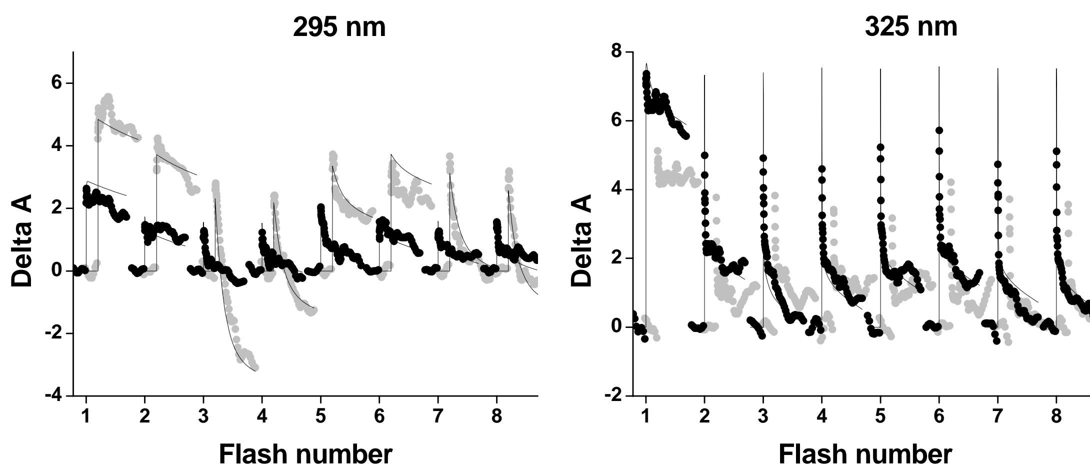

Figure. 5. PSII membranes Ca^{2+} depleted by exposure to 2 M NaCl / 1 mM EGTA as in Fig. 4 but using extensively chelex treated buffers. Grey traces left frame: after addition of 15 mM $CaCl_2$. Grey traces right frame: 325 minus 295 nm. Lines: fits showing that 40 % of PSII was lost and 82 % of the remaining 60 % could only oxidize Y_Z after treatment.

treatment, and are not repaired by the addition of Ca^{2+}. Exposure to room light for 30 min after the 30 min dark incubation made little difference (black traces).

In an effort to minimize Ca^{2+} contamination during the manipulation of PSII samples, the resuspending and washing buffers used in Figure 4 were treated with Chelex to remove as much adventitious Ca^{2+} as possible. Figure 5 shows that the pattern of absorbance changes after NaCl treatment, both at 295 and 325 nm, was now similar to that obtained by the pH 3 / citrate treatment. The important difference, however, is that in these samples the extrinsic PsbP and PsbQ subunits are absent, so Ca^{2+} addition rapidly restores the absorbance oscillation of the S-state cycle (grey traces at 295 nm). The lines show a fit as in Fig. 3, indicating 40 % irreversible loss of PSII reaction center activity, inhibition of S-state advance in 82 % of the rest, with 50 % decay of long-lived Y_Z• between flashes.

3.4 DISCUSSION

The S-state cycle, as monitored by its UV absorbance changes in a series of single-turnover flashes, is a different probe of PSII activity than the steady state rate of oxygen evolution under saturating illumination measured with a Clark electrode. The first few cycles may exhibit robust oscillations in a

sample that shows no significant activity with the Clark electrode, either because relaxation at the electron acceptor side is too slow to support a turnover rate of more than a few electrons per second, or because the sample is rapidly inactivated during illumination, for instance by release of the Ca^{2+} ion in the S_2 and S_3 states. The first possibility cannot explain the fact that the substantial inhibition of oxygen evolution caused by the salt-wash procedure was not reflected in the UV absorbance measurements. Salt-washing does introduce a rate limitation at the acceptor side, but that is not relieved by Ca^{2+} addition (Dekker et al. 1984a). This may explain why reactivation of the S-state cycle by Ca^{2+} observed here is larger than the activity increase that is generally reported for light-saturated oxygen evolution rates, but it cannot explain that activity increase. Therefore the second possibility, inactivation by Ca^{2+} loss during measurement, must be responsible for the inhibition of steady state oxygen evolution. The same conclusion was drawn by Ädelroth et al. (1995) from their finding that the Ca^{2+} ion was present in NaCl-treated PSII membranes.

In the most rigorously Ca^{2+}-depleted condition that could be obtained with the NaCl-wash procedure (Figure 5), the pattern of flash-induced absorbance changes was the same as with the pH 3 / citrate treatment, suggesting that a Ca^{2+} free S_1 state was obtained that is not oxidized to S_2 on flash illumination. This is in agreement with reports in the literature showing that the Ca^{2+} free, stable S_2 state can only be formed if the extrinsic polypeptides are rebound (Boussac et al. 1989) or if a Ca^{2+} chelator is added immediately after illumination at high ionic strength (Ono and Inoue 1990a). Those findings suggest that the Ca^{2+} free S_2 state is destabilized by the residual Ca^{2+} contamination in the medium. To explain why the sample of Fig. 5 was in the Ca^{2+} free S_1 state, such destabilization would have to occur even if the Ca^{2+} concentration is much too low to saturate the binding site in the S_1 state, let alone in the S_2 state.

Perhaps a more likely interpretation might be that the stable S_2 state was accumulated in the salt-washed sample of Fig. 5. In fact, the observed behavior, initial oxidation of Y_Z followed by $P_{680}^+Q_A^-$ recombination on later flashes, is precisely what would be expected to occur if the sample was in the Ca^{2+} free S_2 state. The unexpected result is that the Ca^{2+} free S_1 state in pH 3 / citrate treated PSII behaves this way and does not advance from S_1 to S_2 on

flash illumination at room temperature (Haumann and Junge 1999), not only in PSII core particles but also in BBY membranes that have been reported to do so under continuous illumination (Ono and Inoue 1990b).

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4 The Ca^{2+} requirement for the S_1 to S_2 transition of the oxygen-evolving complex

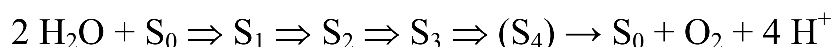
ABSTRACT

The functional role of the Ca^{2+} ion in the oxygen-evolving complex of photosystem II (PSII) is not yet clear. Current models explain why the redox cycle of the complex would be interrupted after the S_3 state without Ca^{2+}, but the literature shows that it is interrupted after the S_2 state. Re-interpretation of the literature on methods of Ca^{2+} depletion (Chapter 2 / Miqyass et al 2007 Photosynth Res 92: 275-287) led us to conclude that not only the oxidation of S_2 to S_3 but also the oxidation of S_1 to S_2 requires Ca^{2+}. Here we confirm this interpretation. Ca^{2+} depletion of PSII membranes by high ionic strength was studied by measuring flash-induced S-state transitions in UV absorbance. Using KCl instead of NaCl, we found that a 30 minute incubation in the dark leaves PSII in an inactive S_1 state, where a flash caused oxidation of the secondary electron donor Y_Z only. However, a 30 min exposure to room light in the presence of KCl and a Ca^{2+} chelator produced the inactive stable S_2 state, in which Y_Z oxidation was also largely inhibited. In the absence of Ca^{2+}, Y_Z• can still oxidize S_1 to S_2 but the flash yield is negligible, which indicates an essential functional role of Ca^{2+} in the S_1 to S_2 transition.

4.1 INTRODUCTION

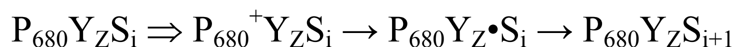
The Ca^{2+} ion in the oxygen-evolving complex (OEC) of photosystem II (PSII) stabilizes Mn ligation (Mei and Yocum 1991, 1992) and this probably plays an essential functional role during the assembly of the Mn_4Ca cluster that constitutes the active site (Tyryshkin et al. 2006). In addition, a surprising variety of mono-, di-, and trivalent metal cations can act as competitive inhibitors of O_2 evolution (Yocum 1991), presumably replacing Ca^{2+} at its binding site but unable to fulfill another essential functional role of the Ca^{2+} ion (e.g. Ono et al. 2001; Vrettos et al. 2001, van Gorkom and Yocum 2005). Only Sr^{2+} can replace Ca^{2+} functionally and the modified properties of that system support proposals that Ca^{2+} plays a role in the mechanism of H_2O oxidation as a Lewis acid that facilitates deprotonation of H_2O. The resulting Ca^{2+}-bound OH^- ion is proposed to attack a Mn^V=O to catalyze O–O bond formation (Pecoraro et al 1998; McEvoy and Brudvig 2004).

However, those models do not explain the inhibition of O_2 evolution at other steps in the mechanism preceding O-O bond formation when Ca^{2+} has been removed. The O–O bond formation reaction takes place after the OEC has accumulated 4 oxidizing equivalents by 4 successive photoreactions (Kok et al. 1970):



$$2\ H_2O + S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow (S_4) \rightarrow S_0 + O_2 + 4\ H^+$$

Each of these S-state transitions is caused by oxidation of the secondary electron donor, tyrosine Z, by the photo-oxidized reaction center chlorophyll P_{680}^+:



$$P_{680}Y_ZS_i \Rightarrow P_{680}^+Y_ZS_i \rightarrow P_{680}Y_Z{\bullet}S_i \rightarrow P_{680}Y_ZS_{i+1}$$

It appears to be generally accepted now that in the absence of Ca^{2+} the S_3 state cannot be formed and the reaction sequence does not proceed beyond the state $P_{680}Y_Z^{\bullet}S_2$.

Most of the relevant literature proposes that Ca^{2+} is not required for S_2 formation, but we recently concluded (Chapter 2 / Miqyass et al. 2007) that the evidence for this hypothesis appears to derive from experiments involving either flash illumination of samples that may have rebound residual Ca^{2+} in low S-states following its dissociation in high S-states, or, alternatively,

samples exposed to continuous illumination that may cause accumulation of S_2 by a slow and inefficient reaction that is functionally irrelevant. The latter explanation could also provide the basis for the results of Lee et al. (2007), who recently reemphasized the point that Ca^{2+} does not play an essential functional role in the S_1 to S_2 transition.

In the work reported here, the function of Ca^{2+} in the S_1 to S_2 transition is characterized using flash-induced UV absorption difference measurements on salt-washed PSII membranes depleted of the PsbP and PsbQ extrinsic subunits. In these preparations, Ca^{2+} was replaced by K^+, which is more efficient than Na^+ in its ability to displace Ca^{2+} (Ono et al. 2001; Nagel and Yocum 2005). The results presented here show that the Ca^{2+}-free S_1 state is not efficiently oxidized to S_2 upon flash illumination, in agreement with similar measurements by Haumann and Junge (1999) on a PSII core preparation (and confirmed for PSII membranes in the previous chapter) that had been Ca^{2+}-depleted by the pH3/citrate method of Ono and Inoue (1988).

4.2 MATERIALS AND METHODS

PS II membranes were prepared from spinach according to Berthold et al (1981) with modifications described in Ghanotakis, et al. (1984), and stored in 0.4 M sucrose, 50 mM MES and 10 mM NaCl. For Ca^{2+} depletion, they were suspended at 0.4 mg of chlorophyll (Chl)/ml in 0.4 M sucrose, 50 mM MES, 30 mM betaine and 30 mM tetramethylammonium chloride, pH 6.0 (buffer A). All subsequent manipulations were done in the dark (unless otherwise noted) and all buffers were treated with Chelex to remove traces of Ca^{2+}.

After stirring for 5 min, an equal volume of 2M KCl and 2mM EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) was added to obtain final concentrations of 0.2 mg Chl/ml, 1M KCl, and 1mM EGTA. The mixture was first incubated for 30 min at 4 °C in the dark and, where indicated, then exposed to room light for 30 min at 4 °C. To assure homogeneous light exposure, the sample was spread in a thin layer on the flat bottom of a large Erlenmeyer flask. The KCl-washed PSII membranes were pelleted by 20 min centrifugation at 48 000 x g and resuspended in buffer A. Based on the dilution ratio alone, the K^+ and EGTA concentrations during measurements are estimated to be about 10 mM and 10 μM, respectively. The

sample was suspended in buffer A at a Chl (*a*+*b*) concentration of 200 μg/ml. As electron acceptors 100 μM DCBQ (2,6-dichloro-p-benzoquinone) and 400 μM ferricyanide ($K_3Fe(CN)_6$) were added. Measurements of UV-absorbance changes and fitting to the Kok model were carried out as described in Chapter 3.

4.3 RESULTS

Figure 1 shows that after exposure of PSII membranes to 1 M KCl / 1 mM EGTA for 30 min at 4 ^oC in the dark (upper frames), the characteristic period 4 oscillation of UV absorbance induced by a series of saturating flashes is observed only when Ca^{2+} is added to the medium (gray circles). At 295 nm (left frames), where contributions by the electron acceptor side of PSII are small and the flash-induced changes are largely due to the S-state cycle, the pattern with Ca^{2+} is normal, apart from the known effects of removing the extrinsic PsbP and PsbQ subunits by high ionic strength treatment: The miss probability is increased due to the much slower S_3 to S_0 transition (the decay observed after flash numbers 3 etc.) and Q_A^- reoxidation (the decay at 325 nm, right frames), and the lifetime of the high S-states is shortened (at 1 flash/s the damping of the oscillation is stronger than at 4 flashes/s). The first flash amplitudes and fits to the Kok model (lines), indicate that nearly all PSII centers advanced from S_1 to S_2 on the first flash.

Without Ca^{2+}, however, the oscillation of absorption changes is suppressed (black circles). The pattern is equivalent to that observed after inactivation of PSII by Tris-treatment: the first flash produced Q_A^- in nearly all centers but the contribution of the donor side is halved at 295 nm and absent at 325 nm, indicating that the oxidized secondary electron donor Y_Z• was unable to oxidize S_1 to S_2**.** From the second flash on short-lived transients at 325 nm are observed that indicate reoxidation of Q_A^- by recombination with P_{680}^+. A minor fraction of Y_Z• was reduced in the 250 ms between flashes.

Exposure to room light for 30 min following the 30 min dark incubation in the presence of 1 M KCl and 1 mM EGTA, leads to almost complete suppression of absorbance changes at 295 nm (lower left frame). At 325 nm a pronounced 0.3 ms $P_{680}^+Q_A^-$ recombination is observed on all flashes including the first, and only a minor fraction of long-lived Q_A^- is seen. An expanded view of the

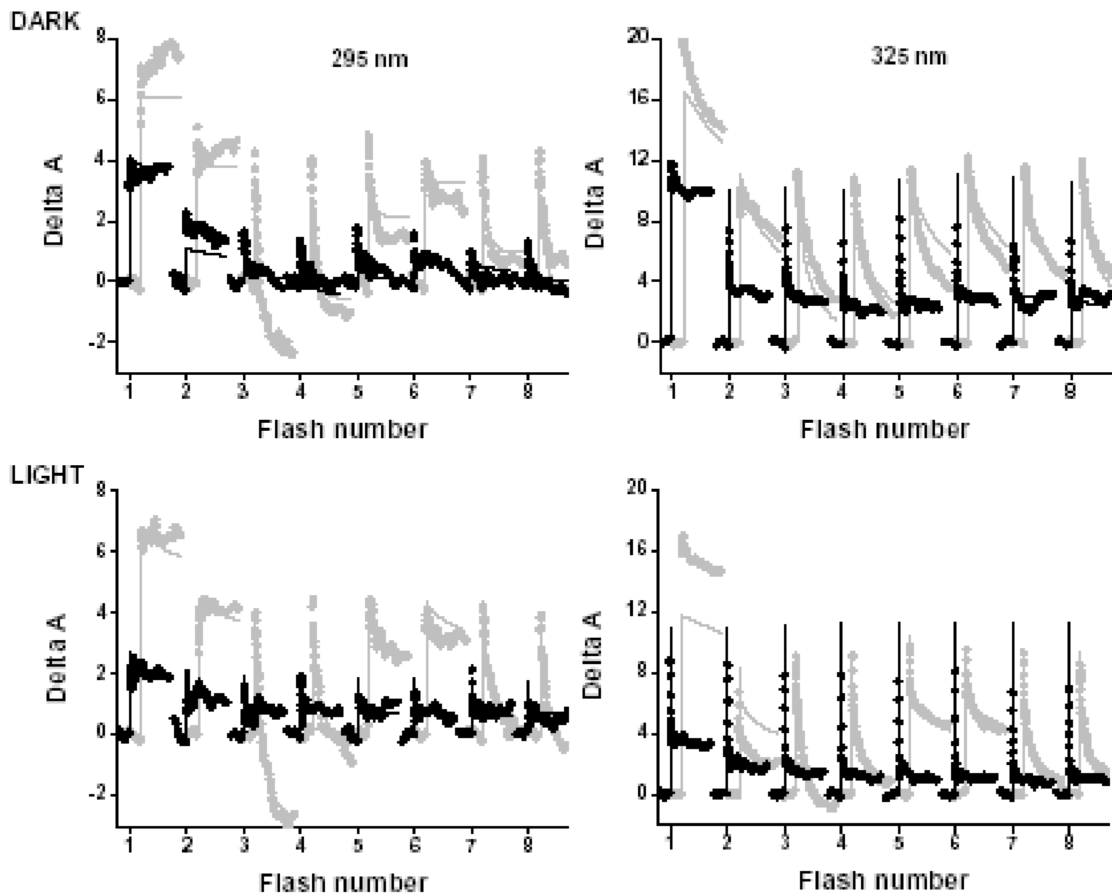


Figure 1A. Absorbance changes at 295 nm and 325 nm induced by a series of saturating flashes in PSII membranes. The preparation was depleted of Ca^{2+} by incubation in 1 M KCl / 1 mM EGTA for either 30 min in the dark (upper frames), or for 30 min in darkness followed by 30 min in room light (lower frames). Gray circles: 15 mM $CaCl_2$ added before measurements. Lines: fits to the Kok model. Flashes were spaced at 250 ms and absorbance was measured from 20 ms before to 50 ms after each flash. The time constant of the fast phase at 325 nm was 0.3 ms. Amplitudes are given in absorbance units $mM^{-1}cm^{-1}$ assuming 250 Chl(*a*+*b*) / PSII.

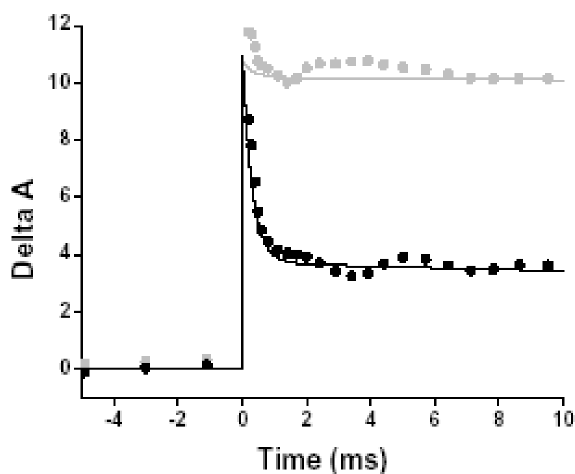


Figure 1B. Expanded view of the first flash at 325 nm in room light exposed (black) and dark treated (grey) Ca^{2+}-free PSII.

first flash trace is shown in Fig. 1B to illustrate the difference with that in the non-illuminated sample (grey). This situation persisted for at least 5 h in samples stored in darkness at 4 ^{o}C**,** and may correspond to the 'stable, modified S_2 state' (Boussac et al. 1989; Sivaraja et al. 1989; Ono and Inoue 1990b). After addition of 15 mM $CaCl_2$ to the medium, the absorption decrease associated with the S_3 to S_0 transition first occurred on the third flash (Fig.1A grey circles), as in the non-illuminated sample, indicating that the sample was in the S_1 state at the start of the flash series, consistent with what would be expected in view of the short lifetime of the higher S-states in these preparations.

In order to determine whether an illuminated, Ca^{2+}-depleted sample was in the S_2 state, measurements were carried out immediately after mixing a high $CaCl_2$ concentration into the measuring cuvette. Although such measurements presented extraordinary difficulties owing to baseline instability, sufficient data could be accumulated using a delay time of 2-3 s between $CaCl_2$ addition

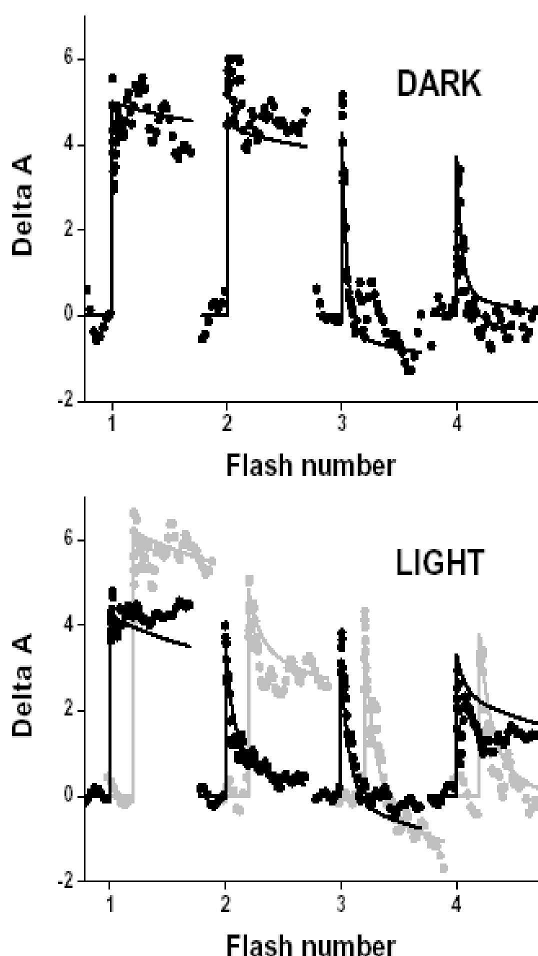


Figure 2. The effect of 70 mM $CaCl_2$ added immediately (2-3 seconds) before a flash series, on absorbance changes at 295 nm. Conditions are given in Fig. 1. Upper frame: KCl-treated in the dark. Lower frame: samples incubated with KCl in darkness and then in room light. Grey traces: Identical conditions, but with a 10 s delay between $CaCl_2$ addition and initiation of the flash series. The lines represent a global fit to the Kok model (see text).

to give a final $CaCl_2$ concentration of 70 mM, and the start of the flash series. Typical results of such an experiment are shown in Figure 2 (black circles). The data show that the absorption decrease at 295 nm on the S_3 to S_0 transition occurred on the second, instead of the third, flash if the preparation had been exposed to room light during KCl treatment. This result confirms that PSII was in an S_2 state at the moment of Ca^{2+} addition. At a delay time of 10 s between $CaCl_2$ addition and the first flash, a major fraction of centers had already decayed to S_1 (grey circles). The lines in Figure 2 show a global fit of these data to the Kok model. Data-set dependent fit variables were the S-state distribution and the fraction of inactive centers at the moment of the first flash. At 3 s after 70 mM $CaCl_2$ addition, 54 ± 5 % of the active centers were in S_2 in the light-exposed preparation, while this fraction was < 5 % in the dark incubated preparation. After a 10 s incubation following $CaCl_2$ addition, more centers had been reactivated, but only 19 ± 5 % were still in S_2. In view of the convolution of the activation by Ca^{2+} binding with the rapid decay of $S_2(Ca^{2+})$ to S_1, the fraction of centers trapped in the stable S_2 state by the room light exposure during KCl treatment was probably much larger than 54 %.

The main difference between the Ca^{2+}–free S_1 and S_2 states in these measurements is the yield of stable charge separation on the first flash (Fig. 1B), where Y_Z is oxidized in the $S_{1'}$ state and $P_{680}{}^{+}Q_A{}^{-}$ recombination occurs in S_2. Since their absorbance changes on later flashes are similar, however, we cannot rule out that $Y_Z{\bullet}S_1$ is slowly converted to Y_ZS_2 during the flash series. To investigate this possibility the amplitude of the long-lived component of the 325 nm absorbance change in a non-illuminated preparation (grey symbols in Figure 3) was also measured with a 15 s delay before the last flash

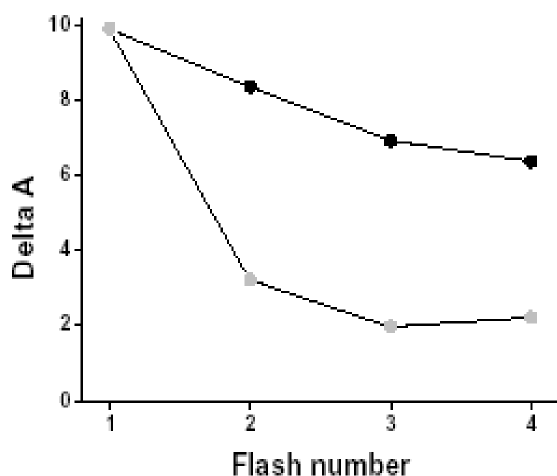


Figure 3. Amplitude of the 'stable' ($\tau >> 0.3$ ms) absorbance change at 325 nm after Ca^{2+} depletion by KCl treatment in the dark. Conditions are as given in Fig. 1. Absorbance changes were measured with (black) or without (grey) a 15 s delay before the last flash to allow complete reduction of $Y_Z{}^{\bullet}$.

to allow complete reduction of Y_Z• (black symbols). This amplitude indeed decreases with increasing flash number, by about 15 % per flash. However, no corresponding appearance of a 0.3 ms transient was observed. If this decrease can somehow be attributed to S_2 formation, its flash yield was approximately 15 %, but otherwise the S_2 yield was much less.

The fact that the stable S_2 state could be trapped in illuminated KCl-treated PSII, whereas NaCl-treated PSII was found to be in the S_1 state under otherwise identical conditions (Chapter 3), may be due to a higher affinity of the Ca^{2+} binding site for K^+ than for Na^+. With KCl it was even possible to remove most of the Ca^{2+} from its binding site without addition of any chelator to the high salt incubation medium, as shown in Figure 4. At 295 nm (left frame) residual period-4 oscillations and S_3 to S_0 transients from the third flash indicate that Ca^{2+} was retained in a significant fraction of the centers, but the 0.3 ms transients at 325 nm (right frame) show nevertheless that most centers were inactivated. Although the sample had been exposed to room light during KCl treatment, the extent of the 0.3 ms decay was rather small on the first flash, suggesting that most of the Ca^{2+}-free PSII was in the S_1 state rather than the S_2 state. Presumably the much larger residual Ca^{2+} concentration, compared to the experiments of Fig.1, had destabilized the $S_2(K^+)$ state and yet was not sufficient to replace K^+ in the S_1 state.

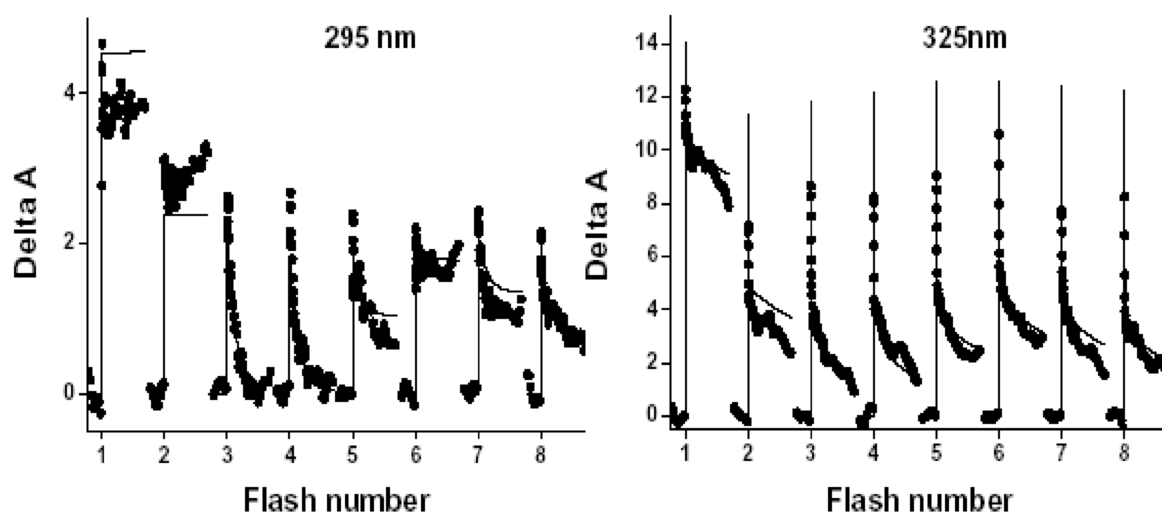


Figure 4. Absorbance changes at 295 and 325 nm after Ca^{2+}-depletion by exposure to 1 M KCl for 30 min dark followed by 30 min room light. Conditions are given in Fig. 1, except that no EGTA was used during Ca^{2+} extraction in these experiments. Lines: fit to Kok model, showing an initial distribution of 25 % active centers, 52 % centers in $S_1(K^+)$, and 23 % centers in $S_2(K^+)$.

4.4 DISCUSSION

These results confirm that K^+ is more effective than Na^+ in removing Ca^{2+} from its functional site in PSII and suggest that, after dissociation of PsbP and PsbQ due to high ionic strength, these cations can replace Ca^{2+} in a binding competition where K^+ shows higher affinity for the site than Na^+. With 1 M KCl, neither a chelator nor room light exposure is essential for extensive Ca^{2+} depletion of PSII, although both are required to accumulate the stable $S_2(K^+)$ state. Trapping the corresponding $S_2(Na^+)$ state requires in addition that the extrinsic polypeptides are rebound (Boussac et al. 1989) or a Ca^{2+} chelator is added immediately after illumination at high ionic strength (Ono and Inoue 1990a).

Using 2 M NaCl without a chelator, Dekker et al. (1984) demonstrated that the S-state cycle was not inactivated unless prolonged illumination (150 saturating flashes) was applied. They also noted, however, that ferricyanide could not be used as an electron acceptor, even when DCBQ was added as well, an observation that we would now attribute to the K^+ added as a counter ion. This issue was addressed earlier by Kimura and Ono (2001), in a discussion of the FTIR study by Noguchi et al. (1995) that indicated Ca^{2+} dependence of the S_1 to S_2 transition in NaCl treated PSII. For this reason, it now seems likely that the inactivation of PSII by illumination in the presence of NaCl and EGTA, reported in Figure 5 of the previous chapter, was actually dependent on the K^+ added with ferricyanide, even though the concentration was only 1.2 mM. Thus we have no unequivocal evidence that Na^+ can replace Ca^{2+} in the S_1 state and suspect that a low concentration of K^+ suffices to replace the functional Ca^{2+} in PSII preparations supposedly 'Ca^{2+}-depleted' by NaCl treatment and thereby inactivate S_1.

In the case of KCl treatment in the dark, like pH3/citrate treatment, it seems clear that Ca^{2+} was removed from its functional site in the S_1 state and that the Ca^{2+}-depleted S_1 state was inactive. The Ca^{2+}-depleted S_1 state differs from the Ca^{2+}-depleted S_2 state in that it allows efficient oxidation of Y_Z after a flash and shows $P_{680}{}^+Q_A{}^-$ recombination only when a subsequent flash is fired within the lifetime of Y_Z• (a few seconds). The flash yield of Y_Z oxidation in the Ca^{2+}-depleted S_2 state, however, is low and $P_{680}{}^+Q_A{}^-$ recombination is observed on the first flash. On the basis of this distinction it is now clear that

Ca^{2+} depletion carried out by illumination of PSII in the presence of 2 M NaCl /1 mM EGTA, as described in the previous chapter, did not produce the stable S_2 state. The observation of a Ca^{2+} free S_1 state does not imply that the Ca^{2+} concentration is too low to destabilize the Ca^{2+} free S_2 state. Since the life time of $S_2(Ca^{2+})$ appears to be only about 10 s in these preparations, a quite low population of that state would be sufficient to explain the loss of S_2 before the start of measurements.

The observation that the stable $S_2(K^+)$ state requires more rigorous Ca^{2+} depletion than $S_1(K^+)$ suggests that $S_2(K^+)$ must have been formed by S-state advance from $S_1(K^+)$ during room light exposure, and not by Ca^{2+} release from a higher S-state as in the case of NaCl treatment. No significant conversion of Ca^{2+}-depleted S_1 to S_2 could be detected after a few flashes, but this conversion is complete within 30 minutes under room light. A time constant between 10 s and 10 min would indicate that the oxidation of S_1 to S_2 by Y_Z• is 5-7 orders of magnitude slower than the 0.1 ms observed in the presence of Ca^{2+}. This would prevent photosynthesis even if it were the only reaction that requires Ca^{2+}, so the metal clearly has an essential functional role in the S_1 to S_2 transition. Perhaps the same kinetic constraint, rather than a decreased potential, is responsible for the fact that S_2 to S_1 decay is slowed down by more than 3 orders of magnitude in the absence of Ca^{2+}.

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5 S-state dependence of accessibility and affinity of the Ca^{2+} binding site

ABSTRACT

The exchange of Ca^{2+} for K^+ and Cd^{2+} was investigated in the successive oxidation states (S-states) of the oxygen-evolving complex of spinach Photosystem II by measurement of UV absorbance changes induced by a series of saturating flashes. The extrinsic PsbP and PsbQ polypeptides were removed, as their presence prevented rapid exchange. The results show that the ratio of binding affinities for Ca^{2+} and K^+ varies by at least a factor of 500 during the S-state cycle with a minimum in the S_3 state of 1 (half inhibition at equal concentrations added), whereas the affinity ratio for Ca^{2+} and Cd^{2+} changed little with S-state. The exchange rates are much higher in S_2 and S_3 than in S_0 and S_1. In the stable $S_2(K^+)$ state a slow (>> 5 s) structural modification takes place that prevents recovery of the slow-exchange situation on return to a low S-state and causes a low flash yield of tyrosine Z oxidation in the $S_2(K^+)$ state, but does not inhibit the S-state cycle in the presence of Ca^{2+}. The implications of these findings for the functional role of Ca^{2+} in the S-state cycle are discussed.

5.1 INTRODUCTION

In order to obtain a Photosystem II (PSII) preparation that depends on Ca^{2+} addition for O_2-evolution activity, the most commonly used method, exposure to a high NaCl concentration requires illumination of the sample (Dekker et al. 1984; Miyao and Murata 1986). The origin of this light-dependence was investigated by Boussac and Rutherford (1988), who measured the effect on O_2-evolution activity of a 10 min. exposure to 1.7 M NaCl in the dark, applied immediately after illumination by a variable number of single-turnover flashes to obtain different initial oxidation states (S-states) of the O_2-evolving complex (OEC). Their results revealed a clear dependence on S-state and showed that NaCl treatment is most effective in the S_3 state.

Reinterpretation of the literature (Miqyass et al. 2007 / Chapter 2) and the results presented in Chapter 3 led to the conclusion that 'Ca^{2+} depletion' by NaCl/light treatment produces a preparation that has Ca^{2+} in its binding site and is functionally competent, unless special precautions are taken to prevent residual Ca^{2+} contamination in the sample from rebinding after the treatment. The fact that NaCl/light treatment nevertheless results in a preparation that requires Ca^{2+} addition for O_2 evolution activity was attributed to a modification of PSII that opens the Ca^{2+} site to rapid exchange (Miyao and Murata 1986). Ca^{2+} loss would then occur in the higher S-states formed during the assay if the Ca^{2+} binding affinity is decreased. This interpretation was supported by the finding that the use of KCl instead of NaCl led to inactivation of the S-state cycle even if no light was applied (Chapter 4), which was attributed to a much higher binding affinity of the Ca^{2+} site for K^+ rather than for Na^+, in agreement with the results of Ono et al. (2001). On the other hand Vrettos et al. (2001), studying the binding competition between Ca^{2+} and various metal ions, found no evidence for inhibition by Na^+ or K^+ even at molar concentrations and prolonged (hours) equilibration times.

The experiments reported here were carried out to investigate the S-state dependence of the accessibility and binding affinity of the Ca^{2+} site and the modifications that occur in the Ca^{2+}-free S_2 state. During the measurement of S-state absorbance changes in a flash series, the ionic composition of the sample can be changed between flashes within a few seconds. In this way the accessibility and binding affinity of the Ca^{2+} site of each of the successive S-

states could be analyzed individually. The results confirm the prediction by Boussac and Rutherford (1988) that both affinity and accessibility of the Ca^{2+} binding site play a role in its S-state dependent properties. The modification of the binding site described by Miyao and Murata (1986) is shown to make the Ca^{2+} site as accessible for rapid exchange in low S-states as it would normally be only in the high S-states.

5.2 MATERIALS AND METHODS

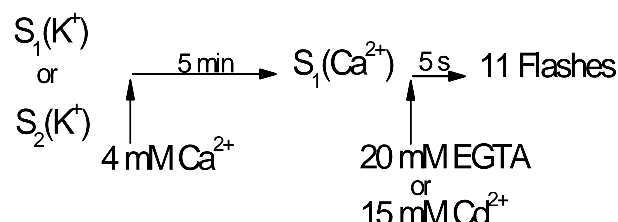
PS II membranes were prepared from spinach according to Berthold et al (1981) with modifications (Ghanotakis et al. 1984) and stored in 0.4 M sucrose, 50 mM MES and 10 mM NaCl. For use, PSII membranes were suspended in 0.4 M sucrose, 50 mM MES, 30 mM betaine and 30 mM tetramethylammonium chloride, pH 6.0 (buffer A). All subsequent manipulations were done in the dark (unless otherwise noted) and all buffers were chelexed to remove residual Ca^{2+}. Extraction of Ca^{2+} was carried out by exposure to 1 M KCl / 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) for 30 min in the dark, or for 30 min in the dark and 30 min under room light, as described in Chapter 4. The KCl-washed PSII membranes after dark or light treatment were in the $S_1(K^+)$ and $S_2(K^+)$ states, respectively (Chapter 4).

Extraction of the PsbP and PsbQ extrinsic polypeptides without Ca^{2+} depletion was done by a 15 minutes incubation in 50 mM Na_2SO_4 and 50 mM HEPES pH 7.5 (Homann 1988; Wincencjusz et al. 1997). For measurements samples were suspended in buffer A at a Chl (*a*+*b*) concentration of 200 µg/ml, and the electron acceptors 100 µM DCBQ (2,6-dichloro-p-benzoquinone) and 400 µM ferricyanide, or 100 µM PPBQ (phenyl para-benzoquinone) were added as indicated in the figure legends.

UV absorbance changes induced by a series of saturating flashes were measured as described in Chapter 3. Flashes were spaced at 250 ms and absorbance was measured from 20 ms before to 50 ms after each flash. Amplitudes are given in absorbance units $mM^{-1}cm^{-1}$ assuming 250 Chl(*a*+*b*)/PSII.

5.3 RESULTS

A series of experiments were carried out to determine the effect of room light exposure, used during the extraction of Ca^{2+} in the presence of KCl, on the properties of Ca^{2+} rebinding to its site in PSII. The strategy used in these experiments is represented diagrammatically below:



Ca^{2+} depleted PSII membranes were prepared in darkness or with room light exposure and were in the state $S_1(K^+)$ or $S_2(K^+)$, respectively, as described in Chapter 4. At least 5 minutes before a measurement 4 mM $CaCl_2$ was added to a sample, converting PSII to the $S_1(Ca^{2+})$ state in both preparations. The S-state absorption changes induced by a series of single-turnover flashes, shown by the grey traces in Figure 1 (same data in upper and lower frames), do not reveal a clear difference between the dark (left) and the light treated (right) preparation. However, as shown by the black traces in Figure 1, when a Ca^{2+} chelator (20 mM EGTA, upper frames) or a competitive inhibitor (15 mM Cd^{2+}, lower frames) was added 5 seconds before the first flash, the S-state cycle was inhibited only in the samples exposed to light during Ca^{2+} extraction. A partial inhibition was obtained with EGTA in 5 s, while the pattern after Cd^{2+} addition suggests that all S_1 was inactivated and any residual activity was due to a small fraction of centers that were initially in the S_0 state or had an unusually high miss probability. The Cd^{2+} concentration used here completely inhibited all S-state transitions in both types of preparations, provided that it was added minutes before measurement. These results clearly confirm the conclusion of Miyao and Murata (1986) that illumination during exposure to high concentrations of NaCl changes PSII to an ‘open’ conformation where Ca^{2+} in the OEC is in rapid exchange with the medium.

In the higher S-states a similar rapid exchange could also be obtained in PSII membranes without Ca^{2+} depletion treatment, provided that the extrinsic PsbP and PsbQ polypeptides had been removed. Figure 2 shows S-state absorbance

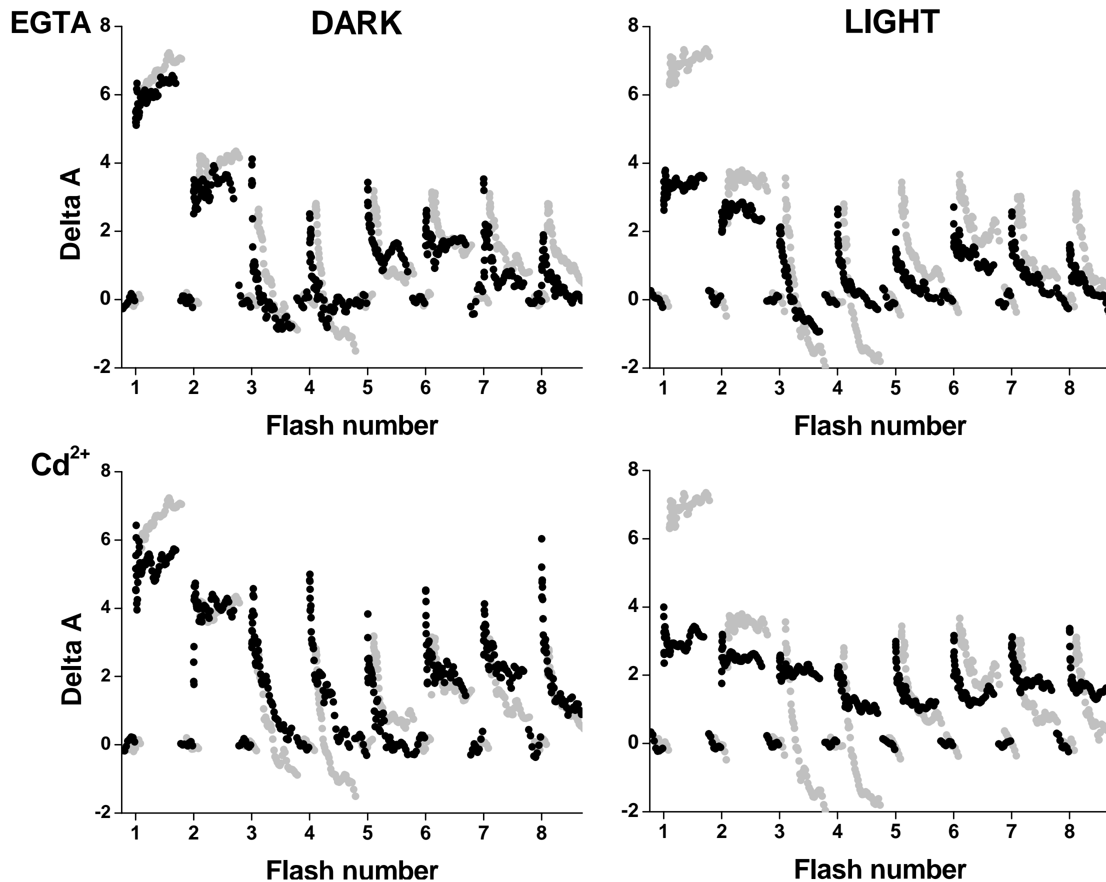


Figure 1. The effect of a saturating concentration of 20 mM EGTA (upper frames, black traces) and 15 mM Cd^{2+} (lower frames, black traces) added 5 seconds before measurement of flash-induced absorbance changes at 295 nm in PSII membranes that had been Ca^{2+}-depleted by exposure to 1 M KCl / 1 mM EGTA either for 30 min in the dark (left frames), or for 30 min in dark and 30 min in room light (right frames), and then reactivated by incubation for at least 5 min with 4 mM CaCl before the experiments (gray traces). Flash frequency 4 Hz. Absorbance changes were recorded from 20 ms before to 50 ms after each flash. Concentrations: 200 µg Chl/ml, in 50mM MES, 0.4 M Sucrose, 30 mM TMAC, 30 mM Betaine, 100 µM PPBQ. Delta A units correspond to differential extinction coefficient $mM^{-1}cm^{-1}$ assuming 250 Chl/PSII.

changes in PSII membranes from which PsbP and PsbQ had been removed by 15 min exposure to pH 7.5 / 50 mM Na_2SO_4 (Homann 1988; Wincencjusz et al.1997), a treatment that avoids both illumination and high ionic strength, and does not extract Ca^{2+}. (Actually this particular preparation had also been exposed for 15 min to 1 mM $CdCl_2$ that was subsequently washed out with 3 mM EGTA, but the treatment failed to remove Ca^{2+} from its binding site in the OEC and should be irrelevant for the measurements described here). In the presence of 15 mM $CaCl_2$, flash-induced absorption changes were measured as in Fig.1, but the flash series was interrupted for Cd^{2+} addition before flash

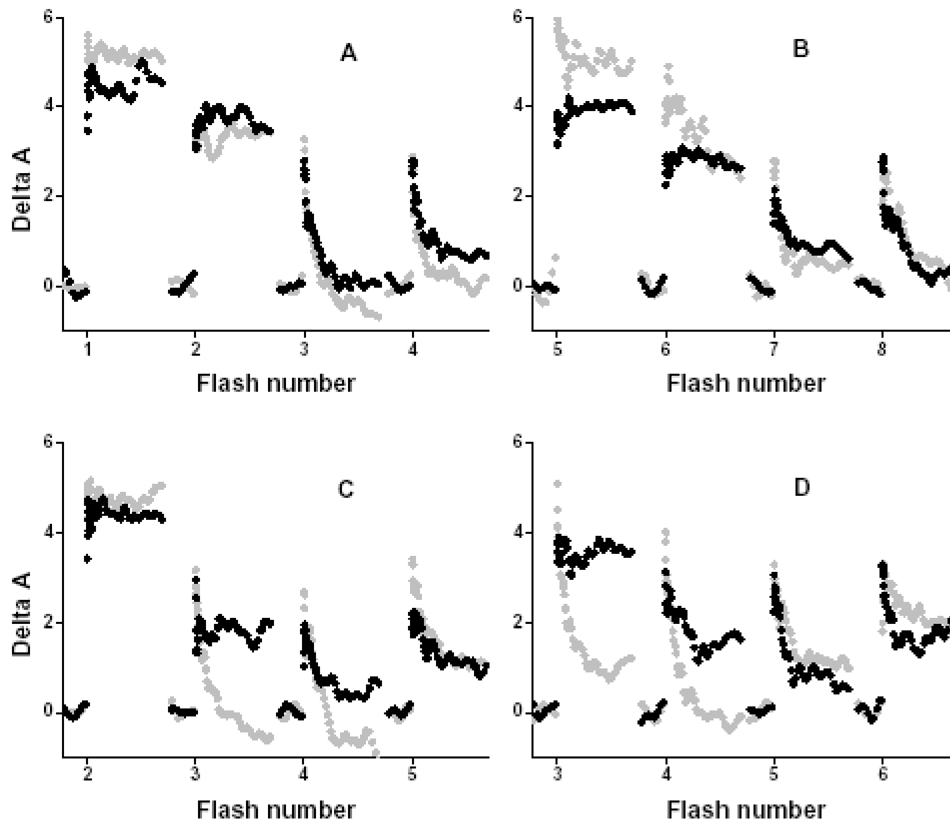


Figure 2. S-state dependence of Cd^{2+} accessibilty and substitution for Ca^{2+} in PSII membranes depleted of PsbP and PsbQ, but not Ca^{2+}, by pH 7.5 / sulfate treatment. Absorbance changes were measured at 295 nm as in Fig. 1. but in the presence of 15 mM $CaCl_2$, and with 100 µM DCBQ as electron acceptor. The first 4 flashes after addition of 5 mM $CdCl_2$ are shown, in A and B (low S-states) 30 s after addition; in C and D (S_2 and S_3) 5 s after addition.

numbers 1, 2, 3, or 5, to probe the Cd^{2+} sensitivity of mainly S_1, S_2, S_3, or S_0/S_1. The traces of the first 4 flashes after Cd^{2+} addition are shown. When added in the S_2 or S_3 state (frame C and D, respectively), Cd^{2+} completely suppressed the absorbance decrease due to the S_3 to S_0 transition on flash number 3**,** within 5 seconds after addition. In low S-states Cd^{2+} had no effect in 5 s (not shown) and even after 30 s, as shown in frames A and B, there was little difference with the control (no addition, shown in grey). The minor effects seen in B are largely due to inactivation of centers that were still in higher S-states after 4 flashes and, in the control, had in part decayed to S_1 during the 30 s delay. The apparent inaccessibility of the low S-states to Cd^{2+} after 4 flashes shows that this is not merely a special property of the dark-adapted state and that the ability of the Ca^{2+} site to undergo rapid exchange is in fact S-state dependent.

The S-state dependence of the ratio of the K^+ and Ca^{2+} binding affinities was investigated in PSII membranes that had been PsbP and PsbQ depleted by the pH 7.5 / sulfate treatment, and then Ca^{2+} depleted by KCl treatment in the dark. These samples did not show S-state absorbance changes in a flash series, but were fully active after a 10 min. incubation with 2 mM Ca^{2+}, as shown by the grey traces in Figure 3A. This means that the residual Ca^{2+} concentration in the sample was much smaller than 2 mM and that the residual K^+ concentration did not significantly compete with 2 mM Ca^{2+} in the dark-adapted state. The approximately 100-fold sample dilution after KCl treatment would suggest a residual K^+ concentration of 10 mM, so the ratio of K^+/Ca^{2+} binding affinities is probably much lower, and that of their apparent dissociation constants (K_d) is much higher, than 5. Different concentrations of K^+ were added during the incubation period and the resulting decrease of the fitted amplitude of the S_3 to S_0 absorption transient on flash number 3 was taken as a measure of the fraction of S_1 that was in the $S_1(K^+)$ state after incubation. The black traces in Fig. 3A show that little inhibition occurred up to a K^+/Ca^{2+} concentration ratio of 50. Half inhibition was only reached near a concentration ratio of 500 (Figure 4).

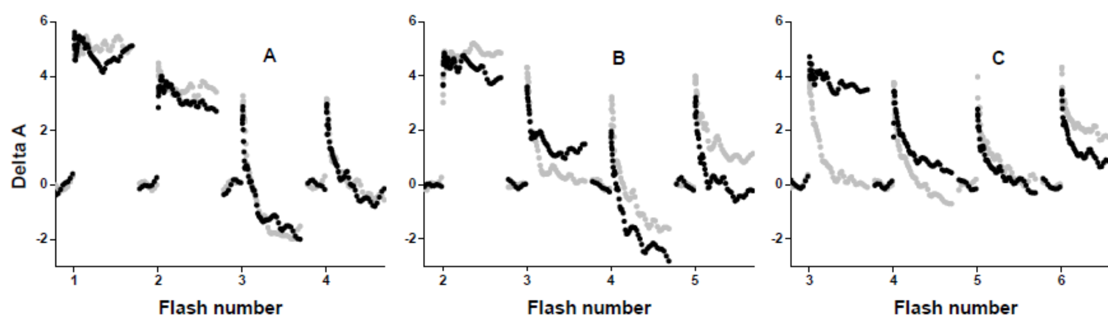


Figure 3. S-state dependence of the binding competition between K^+ and Ca^{2+} in PSII membranes depleted of PsbP and PsbQ by pH 7.5 / sulfate treatment, followed by Ca^{2+} depletion by KCl treatment in the dark. Samples were reactivated by incubation with $CaCl_2$ and then KCl was added 5 min before flash nr 1 (A), or 5 s before flash nr 2 (B), or 3 (C), to determine at which $[K^+]$ / $[Ca^{2+}]$ concentration ratio the substitution occurs in the S_1, S_2, or S_3 state, respectively, as indicated by inhibition of the S_3 to S_0 transition on flash nr 3. $CaCl_2$ and KCl additions in mM: A: 2 and 100; B: 10 and 250; C: 15 and 50. Electron acceptors: 100 µM DCBQ and 400 µM ferricyanide.

For the S_2 and S_3 states similar experiments were carried out, but the K^+ was added after the first or second flash and the flash series was continued after a delay time of 5 seconds. This is much shorter than the life times of S_2 and S_3 in this preparation, which were 34 and 18 s, respectively. If the K^+ was added after 1 flash, the S_3 to S_0 absorption transient on flash number 3 was nearly halved at a K^+/Ca^{2+} concentration ratio of 25 (figure 3B), while a ratio of 4 almost abolished it when added after 2 flashes (Figure 3C). We have not been able to demonstrate any inhibition in S_0, but, as illustrated in Fig. 2B, this involves measurements of the second S_3 to S_0 transition in the flash series, on flash numbers 7 and 8, where the mixture of S-states makes the analysis less reliable.

The extent of inhibition at different K^+/Ca^{2+} ratios, if described by a simple competitive binding equilibrium (lines in Figure 4), indicates K_d ratios of 500, 30, and 1 in the S_1, S_2, and S_3 state, respectively. The K_d ratio in the S_0 state

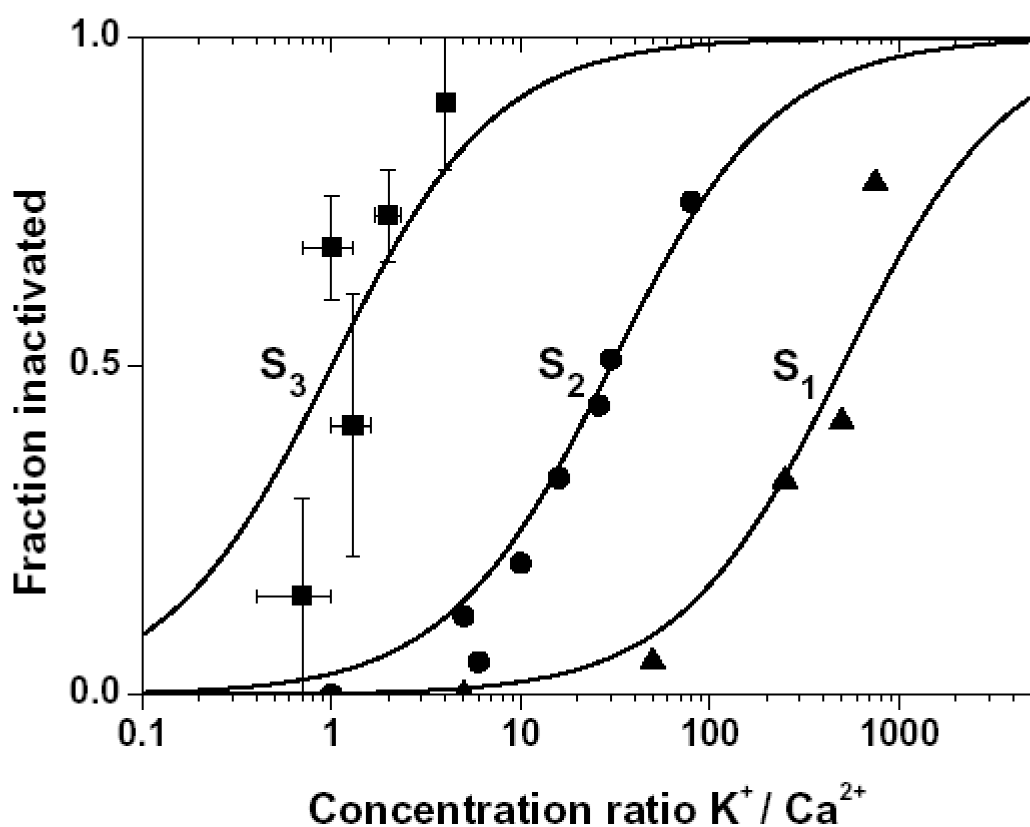


Figure 4. Extent of inhibition versus the ratio of added K^+ and Ca^{2+} concentrations in experiments as shown in Fig. 3. The lines are fits assuming a binding competition at a single site with an S-state dependent K_d ratio, yielding values of 500, 30, and 1 for S_1, S_2, and S_3, respectively. See text for the error bars in the case of S_3.

may be higher than 500. Somewhat erratic results were obtained for S_3 and this cannot be explained by the uncertainty in the assumed 10 mM residual K^+ concentration – and its effect on S_3 in the control data – because that would change all points in the same direction. The error bars shown indicate the effect that an error of ± 5 mM would have. Perhaps more serious is the error due to incomplete equilibration in 5 seconds, but this would shift the S_2 and S_3 curves to even lower K_d ratios and only strengthen the conclusion that the K^+/Ca^{2+} K_d ratio decreases dramatically with increasing S-state. No such pronounced S-state dependence was observed with the divalent competitive inhibitor Cd^{2+}, for which similar experiments indicated half inhibition at Cd^{2+}/Ca^{2+} concentration ratios near 0.2 and 0.1 in S_1 and S_2, respectively.

A remarkable feature of the measurements where an inhibitor was added in the S_2 state, after the first flash, is that the absorbance change on the second flash was not significantly decreased. This may simply be due to the similarity of the differential extinction coefficients for the S_2 to S_3 transition and Y_Z oxidation to Y_Z• at 295 nm (Chapter 3), but the Ca^{2+} free $S_2(K^+)$ state, in contrast to the $S_1(K^+)$ state, was shown in Chapter 4 not to oxidize Y_Z efficiently on flash illumination. Instead, mainly charge recombination in 0.3 ms was observed. Measurements at 325 nm under the conditions used here, however, consistently failed to produce a 0.3 ms transient on the first flash after K^+ addition (not shown). It seems unlikely that K^+ addition induced a fast decay to S_1 or that an S_2 to S_3 transition could still take place. Alternatively, the inhibition of Y_Z oxidation in the $S_2(K^+)$ state may result from a secondary change after K^+ binding that takes more time than the few seconds available in these experiments.

In the case of Cd^{2+} addition after the first flash (Fig.2C), however, the absence of charge recombination on the second flash has a different explanation. Cd^{2+} did not decrease the flash yield of Y_Z• formation in any S-state. As shown in Figure 5, the large 0.3 ms decay of Q_A^- absorbance at 325 nm after the first flash in an $S_2(K^+)$ preparation (the sample was exposed to room light during KCl treatment, trace A) could be suppressed completely by substituting Cd^{2+} for K^+ (trace B). This was not due to S_2 to S_1 decay, which would have occurred in 13 s if Ca^{2+} instead of Cd^{2+} had been added to the sample. The 0.3 ms transient on the first flash reappeared when K^+ replaced Cd^{2+} using KCl and EGTA additions 5 min after Cd^{2+} addition to a sample (trace C). The

$S_1(K^+)$ state does not does not exhibit the 0.3 ms transient on the first flash (Chapter 4).

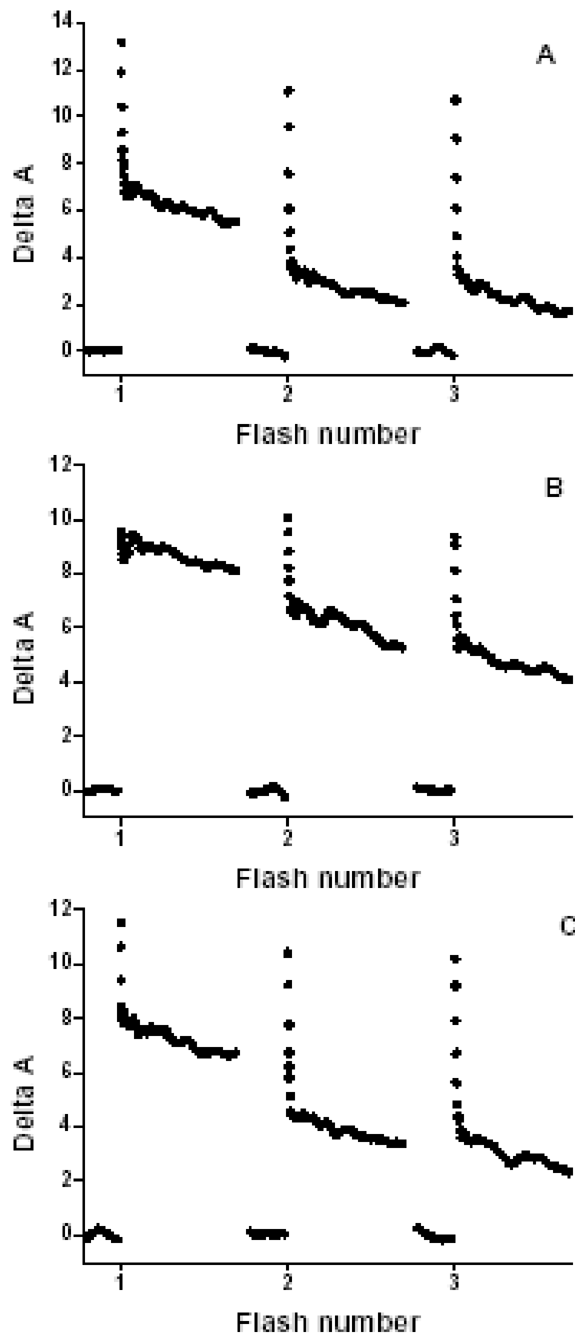


Figure 5. Reversible suppression of $P_{680}^+Q_A^-$ recombination on the first flash by substitution of Cd^{2+} for K^+ in the stable, modified $S_2(K^+)$ state. PSII membranes were Ca^{2+} depleted by KCl treatment with room light exposure. Flash-induced absorbance changes were measured at 325 nm in the presence of 100 μM DCBQ and 400 μM ferricyanide as electron acceptors. B: 5 min after addition of 250 μM $CdCl_2$. C: Incubated for 5 min with 250 μM $CdCl_2$ and then for 5 min with 1 mM EGTA and 10 mM KCl

5.4 DISCUSSION

5.4.1 Accessibility and affinity

Current models of the Mn_4Ca cluster position Ca^{2+} as a structural element sharing carboxylate ligands with 2 or 3 of the Mn ions (Loll et al. 2005; Yano et al. 2006). Such a structure would explain why high concentrations of competing metals or low pH can remove Ca^{2+} from its site, and why other metals of similar charge and ionic radius can reversibly replace it. For concentrations of Ca^{2+} or Cd^{2+} in the low mM range typical binding times were found to be more than an hour for intact PSII, a few minutes after removal of the extrinsic PsbP and PsbQ polypeptides, and a few seconds in high S-states. The release of Ca^{2+} from its binding site involves two different processes. The initial loss of coordinating protein ligands is presumably dependent only on thermal fluctuation and inherent properties of the binding site. If the rate of this process ultimately limits the inhibition kinetics, the maximum rate of inhibition (or reactivation of the inhibited system) could provide useful information about the binding site. However, rate limitation by this process was probably never approached in the experiments reported here, because the rate of inhibition, or reactivation, appeared to be proportional to the concentration of a competing metal, or Ca^{2+}, present in a sample. The second process involved in Ca^{2+} release is its replacement by another species that donates at least one positive charge to stabilize the negatively charged ligand environment of the vacant Ca^{2+} binding site. Under experimental conditions that do not include any substituting cation, the release of Ca^{2+} takes tens of hours (Ädelroth et al. 1995), which might be accounted for by the H^+ concentration if protonation of a ligand is required, because Ca^{2+} is known to be released within 5 min at pH 3 (Ono and Inoue 1988).

The time required for cation substitution at the Ca site appears to be reduced by 1-2 orders of magnitude in the S_2 and S_3 states, as compared to that in S_0 and S_1. This is likely due to a structural change on the S_1 to S_2 transition, for example the dissociation of a carboxylate ligand from Ca^{2+} (Noguchi et al. 1995), which is reversed when the cycle proceeds to a low S-state again. The increased positive charge caused by oxidation of a Mn linked to Ca^{2+} by a bridging carboxylate would be expected to substantially increase Ca^{2+} release. This could explain both the much faster Ca^{2+} exchange with other divalent

cations (Fig. 2) and the drastic change in the relative affinities of the binding site for monovalent and divalent cations (Fig. 4). Na^+ can bind in the high S-states, but the electrostatic repulsion between the ligands prevents binding of Na^+ in the S_1 state, as emphasized by Vrettos et al. (2001). This allows binding of K^+ in the S_1 state, presumably because K^+ with a ~1.4 Å ionic radius is larger than either Na^+ or Ca^{2+}, whose ionic radii are about 1 Å, but K^+ exhibits a 500 times lower affinity than Ca^{2+} in the S_1 state, while in the S_3 state the affinities for K^+ and Ca^{2+} are equal. It should be noted that the K^+/Ca^{2+} concentration ratios referred to here are based on the concentrations added. The dissociation constant K_d is the activity of the ion at which the binding site is half occupied, in the absence of other binding species. Since most of the Ca^{2+} ions, but not of the K^+ ions, may be bound non-specifically to the membranes (Stevens and Lukins 2003; Kreimer et al. 1988), the actual K_d ratios for K^+/Ca^{2+} may be much higher than the apparent values given here.

The chelator EGTA did not seem to influence the rate of Ca^{2+} release, although its affinity for Ca^{2+} is much higher than that of the Ca^{2+} binding site. Inhibition of the S-state cycle by EGTA could be explained by the decrease of the external Ca^{2+} concentration relative to that of any competitive inhibitor (or H^+) present in the sample, and the rate of inhibition was independent of the EGTA concentration. Since it apparently cannot approach the Ca^{2+} binding site close enough to influence the rate of Ca^{2+} release, it is a useful tool to study the affinity or binding rate of ions that can. In the S_1 state, incubation for 30 min. with EGTA had no effect in the presence of 2 M Na^+ (Chapter 3), which demonstrates that Na^+ cannot bind in S_1. In the 'open conformation' EGTA caused half inhibition in 5 seconds in a sample where the only potential competitive inhibitor was the estimated 10 mM K^+ remaining after KCl treatment (Fig. 1). Apparently, K^+ replaces Ca^{2+} with a rate constant of 20 $M^{-1}s^{-1}$ in the S_1 state if the preparation has been Ca^{2+} depleted by accumulation of the stable S_2 state at high salt concentration under room light. This is 7 times faster than Ca^{2+} binding after removal of PsbP and PsbQ in the dark. Much of the literature on Ca^{2+} depleted PSII involves preparations that were exposed to light during high salt treatment and therefore must have been in this modified state.

5.4.2 *The modified, 'open' conformation of the PSII Ca^{2+} binding site*

Presumably the weaker Ca^{2+} binding in high S-states - possibly due to the dissociation of a carboxylate ligand from Ca^{2+} - is a normal property of intact PSII. This would be of little or no consequence in the presence of PsbP and PsbQ, but when Ca^{2+} is replaced by a monovalent cation (K^+, Na^+) in that situation, a secondary structural rearrangement takes place - possibly a movement of the amino acid providing that carboxylate ligand - that prevents rapid recovery of the native structure when Ca^{2+} rebinds and the complex returns to the S_1 state. The data of Miyao and Murata (1986) suggest that the rearrangement takes 20 minutes and its reversal in the presence of Ca^{2+} takes about an hour. In the experiments of Boussac and Rutherford (1988) the rearrangement was largely complete in 10 min when all center were in a high S-state.

This rearrangement by itself does not inhibit the S-state cycle; it is only the absence of Ca^{2+} (or Sr^{2+}) that prevents S-state advance and dark deactivation of S_2 to S_1. However, the reorganized structure leaves the Ca^{2+} binding site open to rapid exchange. As a result, O_2 evolution will be inhibited by the accumulation of Ca^{2+}-free inactive high S-states during illumination. The physiological concentration ratio $[K^+]$ / $[Ca^{2+}]$ probably exceeds the K_d ratio in S_3, so the presence of the extrinsic polypeptides is probably essential to avoid such inhibition. A similar conclusion was drawn earlier for the essential Cl^- ion in the OEC (Wincencjusz et al. 1998).

The reorganization of the Ca^{2+} binding site is reflected in other properties of the system as well, such as a 2-fold shorter lifetime of the higher S-states in the presence of Ca^{2+}. The flash yield of Y_Z oxidation in the $S_2(K^+)$ state was decreased to 20-30 % (Chapter 4), similar to the finding reported by Andréasson et al. (1995) for $S_2(Na^+)$. Since this effect did not appear within 5 s after replacing Ca^{2+} by K^+, it is not a direct consequence of metal ion substitution and might in fact provide a useful probe to measure the kinetics of the structural rearrangement. We have no evidence that it occurs in S_1 or can be induced by Ca^{2+} depletion in the S_1 state, either by KCl treatment (Chapter 4) or by pH3/citrate treatment (Chapter 3). However, Haumann and Junge (1999) did observe a similar low flash yield of Y_Z oxidation in

pH3/citrate treated PSII core particles. The effect was much larger than can be explained by the slower Y_Z• oxidation in that system, attributed to a pK_a shift from 6 to 8.5 of the proton acceptor normally involved in the reaction, because this reaction was still an order of magnitude faster than $P_{680}^+Q_A^-$ recombination. Since the effect could be reversibly suppressed by replacing K^+ with Cd^{2+} (Figure 5), it probably has to do with the charge deficit in the Ca^{2+} site, but not with the essential function of Ca^{2+} in Y_Z• reduction by Mn, because both Cd^{2+} and K^+ inhibit all S-state transitions (except perhaps S_0 to S_1).

The experiment of Figure 5 also shows that the extreme stability of the Ca^{2+}-free S_2 state, on the contrary, cannot be attributed to the charge deficit in the Ca^{2+} site, as might have been expected because the net charge of the cluster would be the same as in the normal $S_1(Ca^{2+})$ state. Instead, the decay of S_2 to S_1, like the advance of S_1 to S_2, appears to depend specifically on Ca^{2+}, as proposed in Chapter 4. Since Ca^{2+} binding restores these reactions immediately, but takes an hour to convert the open conformation of its binding site back to the normal state (Miyao and Murata 1986), the stability of the Ca^{2+}-depleted S_2 state is probably also instantaneous and not induced by the conformational change.

5.4.3 Function of Ca^{2+}

The data presented in this thesis confirm the interpretation of the literature on Ca^{2+} depletion proposed in Chapter 2 and show that the specific requirement for Ca^{2+} is in electron transfer from the Mn cluster to Y_Z• on each of the S-state transitions, with the possible exception of the S_0 to S_1 transition for which conclusive evidence of Ca^{2+} dependence is still lacking. The cation specificity of this requirement suggests stringent limits on the K_d for dissociation of a proton from a cation-ligated H_2O (Vrettos et al. 2001). Y_Z• reduction requires a protonation step. Oxidation of the Mn cluster may well require deprotonation of a ligand (e.g. Baldwin and Pecoraro 1996). If Ca^{2+} is situated in between Y_Z and Mn (Loll et al. 2005), a H-bond network connecting donor and acceptor via a Ca^{2+}-bound H_2O seems a likely possibility to mediate proton-coupled electron transfer from Mn to Y_Z. In addition, the same H_2O might be involved in O–O bond formation on the S_3 to S_0 transition, but the specific Ca^{2+} requirement for O_2 evolution is apparent in

the S_1 to S_2 and S_2 to S_3 transitions as well. Especially its requirement for the S_1 to S_2 transition, which is not accompanied by H^+ release, may support the view that Y_Z• reduction is coupled to Ca^{2+}-mediated proton transfer from the Mn cluster and does not retrieve the proton from the base that is protonated during Y_Z oxidation, as proposed by Dau and Haumann (2007). Styring et al. (2003) reported that the inhibition of the S_2 to S_3 transition is somewhat alleviated at low pH, suggesting a failure of the Ca^{2+}-free cluster to provide the proton required in the reduction of Y_Z•, rather than its failure to deprotonate an Mn ligand. This is not readily understood if Y_Z oxidation in the same system is inhibited because its normal proton acceptor is already protonated, as concluded by Haumann and Junge (1999). It is tempting to speculate that, somehow, the proton donor for Y_Z• reduction cannot be the base that acts as proton acceptor during Y_Z oxidation, so that the system actually functions as a proton pump. At this stage, however, we can only conclude that current hypotheses do not fully explain the data.

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Summary

Photosynthesis produces carbohydrates by reduction of CO_2 using light as the energy source and H_2O as the ultimate source of electrons, releasing O_2 as a waste product. Oxidation of H_2O by photosystem II is a unique redox reaction in that it requires Ca^{2+} as well as Cl^- as obligatory cofactors of the reaction, which is catalyzed by Mn atoms. The properties of the Ca^{2+} binding site in the O_2 evolving complex and its functional role in this reaction have been addressed in hundreds of publications but nevertheless continue to be the subject of debate.

The literature survey in Chapter 2 summarizes current insights and proposes that some of the confusion may result from misinterpretation of the most often used method to obtain Ca^{2+}-free photosystem II preparations, the 'salt-wash' procedure. It is concluded that this method normally does not remove the Ca^{2+} ion and the widely held belief that Ca^{2+} is not required for the first oxidation step of the complex, the S_1 to S_2 transition, is therefore probably unjustified. The consequences of this view for the interpretation of many observations in the literature are discussed and an analysis of the evidence for Ca^{2+} involvement in the successive steps, 'S-state transitions', of the redox cycle of the complex is presented. The Ca^{2+} ion is proposed to be essential on every step for electron transfer from the Mn cluster to the secondary electron donor Y_Z (tyrosine Z).

Chapter 3 introduces the method used in this thesis to investigate the Ca^{2+} dependence of the S-state cycle by measurement of the associated UV absorbance changes in a series of saturating flashes. The results of Ca^{2+} depletion by the two commonly used procedures, salt-washing and low pH treatment, are compared. The results support the proposal in Chapter 2 that rebinding of the extrinsic PsbP and PsbQ polypeptides or the presence of a Ca^{2+}-chelator after the treatment is essential to avoid reactivation of PSII by rebinding of residual Ca^{2+} to samples that have been depleted of the metal. In those samples that were inactivated by Ca^{2+} depletion, also the S-state transition on the first flash appeared to be inhibited.

The use KCl rather than NaCl for Ca^{2+} depletion by the salt-wash procedure was found to be much more effective, as described in Chapter 4. By applying this method in the dark, samples could be prepared in the Ca^{2+}-free S_1 state,

while illumination during the KCl treatment led to samples that were in a stable Ca^{2+}-free S_2 state. Both samples were incapable of further S-state transitions in a flash series and in the $S_2(K^+)$ sample also the oxidation of Y_Z was largely inhibited. In the absence of Ca^{2+}, Y_Z• can still oxidize S_1 to S_2 but the flash yield is negligible, which indicates an essential functional role of Ca^{2+} in the S_1 to S_2 transition, as proposed in Chapter 2.

Chapter 5 describes a study on the exchange of Ca^{2+} for K^+ and Cd^{2+} in the successive S-states of the O_2-evolving complex. The extrinsic PsbP and PsbQ polypeptides were removed, as their presence prevented rapid exchange. The results show that the ratio of binding affinities for Ca^{2+} and K^+ varies by at least a factor of 500 during the S-state cycle, whereas the affinity ratio for Ca^{2+} and Cd^{2+} changed little with S-state. The exchange rates are much higher in S_2 and S_3 than in S_0 and S_1. In the stable $S_2(K^+)$ state a slow structural modification takes place that prevents recovery of the slow-exchange situation on return to a low S-state and causes the low flash yield of Y_Z oxidation in the $S_2(K^+)$ state mentioned above, but does not inhibit the S-state cycle in the presence of Ca^{2+}. The implications of these findings for the possible mechanism of the role of Ca^{2+} in the S-state cycle are discussed.

Samenvatting

De fotosynthese produceert koolhydraten door reductie van CO_2, waarbij licht als energiebron en H_2O als uiteindelijke electronenbron worden gebruikt, terwijl O_2 vrijkomt als afvalproduct. De oxidatie van H_2O door fotosysteem II is een unieke redoxreactie, gekatalyseerd door een 4-Mn cluster, die zowel Ca^{2+} als Cl^- als essentiële cofactoren vereist. De eigenschappen van de Ca^{2+}-bindingsplaats in het O_2-ontwikkelend complex en de functie van Ca^{2+} in deze redoxreactie zijn reeds in honderden publicaties besproken, maar blijven onderwerp van discussie.

Het literatuuroverzicht in hoofdstuk 2 geeft een samenvatting van de huidige inzichten en stelt voor dat een deel van de verwarring het gevolg kan zijn van een verkeerde interpretatie van de meest gebruikte methode om Ca^{2+}-vrije fotosysteem II preparaten te maken, de 'zout-was' procedure. Deze methode lijkt het Ca^{2+} ion gewoonlijk niet te verwijderen en de wijdverbreide opvatting dat Ca^{2+} niet nodig is voor de eerste oxidatiestap van het complex, de $S_1 \rightarrow S_2$ overgang, is dan niet gerechtvaardigd. De gevolgen die deze conclusie zou hebben voor de interpretatie van vele literatuurgegevens worden besproken en de argumenten voor de betrokkenheid van Ca^{2+} bij de opeenvolgende S-overgangen worden geanalyseerd. Dit leidt tot het voostel dat Ca^{2+} in alle S-toestanden een essentiële rol speelt bij de electronoverdracht van het Mn cluster naar de secundaire electrondonor Y_Z (tyrosine Z).

Hoofdstuk 3 beschrijft de in dit proefschrift gebruikte methode om de rol van Ca^{2+} in de redoxcyclus te onderzoeken door meting van de daardoor veroorzaakte UV-absorptieveranderingen bij belichting door een serie verzadigende laserflitsen. Het effect van de twee gebruikelijke methoden om het Ca^{2+} te verwijderen, de zout-was methode en de lage pH behandeling, wordt vergeleken. De resultaten ondersteunen de conclusie in hoofdstuk 2 dat het systeem na deze behandelingen gereactiveerd wordt door binding van achtergebleven of als verontreiniging geïntroduceerde Ca^{2+} ionen, tenzij binding van de extrinsieke eiwitten PsbP en PsbQ of toevoeging van een Ca^{2+}-chelator dat verhinderen. In omstandigheden waar de redoxcyclus wel onderdrukt was door Ca^{2+} gebrek, was dat bij de eerste flits al zichtbaar.

De zout-was procedure bleek veel effectiever als KCl werd gebruikt in plaats van NaCl, zoals in hoofdstuk 4 wordt beschreven. Toegepast in het donker

levert deze methode een preparaat op, dat zich in de Ca^{2+}-vrije S_1 toestand bevindt, terwijl belichting tijdens de KCl behandeling leidt tot een preparaat in de abnormaal stabiele, Ca^{2+}-vrije S_2 toestand. In beide gevallen treden geen verdere S-overgangen op onder flitsbelichting, terwijl in het $S_2(K^+)$ preparaat bovendien de oxidatie van Y_Z geremd is. Y_Z• kan nog wel S_1 oxideren tot S_2, maar het rendement per flits is verwaarloosbaar. Dit bevestigt dat Ca^{2+} een essentiële rol speelt in de $S_1 \rightarrow S_2$ overgang, zoals in hoofdstuk 2 voorgesteld.

Hoofdstuk 5 beschrijft een onderzoek naar de uitwisseling van Ca^{2+} met K^+ of Cd^{2+} in de verschillende oxidatietoestanden van het complex. De extrinsieke eiwitten PsbP en PsbQ werden verwijderd, omdat zij een snelle uitwisseling verhinderen. De resultaten laten zien, dat de verhouding van de bindingsaffiniteiten voor Ca^{2+} en K^+ tijdens de redoxcyclus varieert met tenminste een factor 500, terwijl die voor Ca^{2+} en Cd^{2+} weinig verandert. De snelheid van uitwisseling is veel hoger in S_2 en S_3 dan in S_0 en S_1, maar in de stabiele $S_2(K^+)$ toestand treedt een langzame structurele verandering op, die verhindert dat het systeem bij terugkeer naar een lagere oxidatietoestand ook weer een langzame uitwisseling gaat vertonen. Deze verandering leidt tot de bovengenoemde remming van Y_Z oxidatie in de $S_2(K^+)$ toestand, maar belemmert de redoxcyclus niet in aanwezigheid van Ca^{2+}. De betekenis van deze bevindingen voor het mogelijke mechanisme van de rol van Ca^{2+} in de redoxcyclus van het O_2-ontwikkelend complex wordt besproken.

Résumé

La Photosynthèse permet aux plantes de produire des hydrates de carbone par réduction du dioxyde de carbone (CO_2) en utilisant l'énergie solaire et l'eau (H_2O) comme ultime source d'électrons. Il en résulte une émission d'oxygène (O_2). L'oxydation de l'eau par le photosystème II (PSII) est une réaction d'oxydo-réduction remarquable dans laquelle l'ion Ca^{2+} et l'ion Cl^- sont des cofacteurs indispensables à cette réaction, catalysée par un groupe de 4 ions de manganèse (Mn). Les propriétés du site de fixation du Ca^{2+} au sein du complexe émetteur d'oxygène (Oxygen Evolving Complex, OEC) et le rôle fonctionnel du Ca^{2+}dans cette réaction ont été décrites dans des centaines de publications mais ce problème demeure un sujet de controverse.

Le chapitre 2 résume l'état actuel des connaissances concernant le rôle du calcium dans l'OEC et propose qu'une partie de la controverse résulte d'interprétations erronées des résultats obtenus par la méthode la plus utilisée (exposition à de hautes concentrations en NaCl 'salt-wash') pour obtenir des échantillons de PSII appauvris en Ca^{2+}. Nous proposons que ce protocole ne conduit pas à l'extraction des ions Ca^{2+}. L'opinion largement répandue selon laquelle le Ca^{2+} n'est pas nécessaire pour la première étape de l'oxydation du OEC : la transition de l'état S_1 à l'état S_2, est donc probablement erronée. Les conséquences de cette proposition pour l'interprétation de nombreuses observations dans la littérature sont énumérées. Nous analysons ensuite l'implication du Ca^{2+} dans les étapes d'oxydations successives des états S du cycle redox du OEC, 'S-state transitions'. Il en résulte que l'ion Ca^{2+} est essentiel à chaque étape du transfert d'électrons entre le cluster de Mn et le donneur secondaire d'électrons : la tyrosine Z.

Le chapitre 3 introduit la méthode utilisée dans cette thèse pour étudier le rôle du Ca^{2+}dans les différents états S du cycle rédox, par la mesure des changements d'absorption dans la région UV au cours d'une série d'éclairs brefs et saturants. Les comportements d'échantillons appauvris en Ca^{2+} par les deux protocoles couramment utilisés, 'salt-wash' et exposition à faible pH, sont comparés. Les résultats obtenus confortent les propositions faites dans le chapitre 2. Dans les échantillons de PSII appauvris en Ca^{2+}, la réinsertion des polypeptides extrinsèques PsbP et PsbQ ou la présence d'un chélateur de Ca^{2+} (EGTA) après le traitement sont essentiels pour éviter la réactivation du PSII

par fixation du Ca^{2+} résiduel présent dans les échantillons. Dans ces échantillons inactivés par extraction du Ca^{2+}, les transitions d'états S après le premier éclair semblent être également inhibées.

L'utilisation de KCl plutôt que du NaCl pour appauvrir un échantillon en Ca^{2+} lors du 'salt-wash' s'est avérée beaucoup plus efficace, ceci est décrit dans le chapitre 4. En appliquant cette méthode à l'obscurité, des échantillons peuvent être préparés dans l' état S_1 sans Ca^{2+}, $S_1(K^+)$, tandis que l'illumination pendant le traitement en présence de KCl conduit à des échantillons qui sont dans un état S_2 sans Ca^{2+}, $S_2(K^+)$. Les deux états ainsi formés ne peuvent prendre part à des transitions d'états. Dans l'échantillon $S_2(K^+)$ l'oxydation de la tyrosine Yz est largement inhibée. En l'absence de Ca^{2+}, Yz• peut encore oxyder S_1 en S_2 mais le rendement est négligeable, ce qui indique que l'ion Ca^{2+} a un rôle fonctionnel essentiel dans la transition de l'état S_1 à S_2, telle que proposée dans le chapitre 2.

Le chapitre 5 décrit une étude sur l'échange du Ca^{2+} avec le K^+ et le Cd^{2+} durant les transitions d'états S successifs de l'OEC. Les polypeptides extrinsèques PsbP et PsbQ ont été supprimés, car leur présence empêchait un échange rapide. Les résultats montrent que le rapport des affinités entre le Ca^{2+} et le K^+ varie d'au moins un facteur 500 au cours du cycle des états S , tandis que le rapport d'affinité entre le Ca^{2+} et le Cd^{2+} est peu modifié. Les taux d'échange entre ions sont beaucoup plus élevés dans les états S_2 et S_3 que dans les états S_0 et S_1. Une lente modification structurelle est observée dans l'état stable $S_2(K^+)$, de ce fait, les échanges ioniques sont toujours rapides lorsque l'on revient à un état S inférieur. Cette modification structurelle est aussi à l'origine d'un faible rendement d'oxydation de la tyrosine Yz dans l'état $S_2(K^+)$, mais ne fait pas obstacle au cycle des états S en présence de Ca^{2+}. L'implication de ces résultats concernant le rôle fonctionnel du Ca^{2+} dans le cycle des états S est ensuite abordée.

Curriculum Vitae

I was born on May 1st 1978 at Ouled si Lahcen (Morocco). In 1996, I obtained a scientific 'Baccalauréat' and in 1998 the 'Diplôme d'Etude Universitaire Géneral' with specialization in Biology at the faculty of Science of the University of Orléans. In 2000, I obtained a Bachelor's degree in Biochemistry with specialization in organic chemistry and I completed my undergraduate studies with a 'Maîtrise de Biochimie Structurale' in 2001on the subject entitled "molecular modeling of the HIV protease: test of inhibitors using docking and (ORAL) mechanics simulator and energy minimizer", under the supervision of Dr. K. Zimmermann in the INRA Center at the Laboratory of Mathematics Informatics and Genome, Versailles (France).

In 2002, I obtained my Master's degree in Molecular Biophysics at the University Pierre et Marie Curie Paris VI (France) on the subject entitled: 'adaptation of a protein folding recognition method based on threading to G protein – coupled receptors; evaluation of the accuracy (specificity/sensitivity) using TM- topology database', under the supervision of Dr. J-F Gibrat in the INRA Center at the Laboratory of Mathematics Informatics and Genome, Versailles (France).

In January 2003, I started my PhD studies under the guidance of Dr. H. J. van Gorkom, at the Biophysics department in the Leiden Institute of Physics (LION).

The results of this work were presented at national and international conferences in the form of posters, notably at the 13th International Congress on Photosynthesis (Montreal, Canada), the 14th International Congress on Photosynthesis (Glasgow, Scotland) and the annual Dutch meeting on Molecular and Cellular Biophysics (Lunteren, The Netherlands).

Since January 2008, I joined the department of corrosion and antifouling at TNO Science and Industry (Den Helder, the Netherlands).

Nawoord

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