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ARTICLE *in* BIOCHEMISTRY · AUGUST 2008

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S-State Dependence of the Calcium Requirement and Binding Characteristics in the Oxygen-Evolving Complex of Photosystem II[†]

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Received April 6, 2008; Revised Manuscript Received June 10, 2008

ABSTRACT: The functional role of the Ca^{2+} ion in the oxygen-evolving complex of photosystem II 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. Reinterpretation of the literature on methods of Ca^{2+} depletion [Miqyass, M., van Gorkom, H. J., and Yocum, C. F. (2007) *Photosynth. Res. 92*, 275−287] led us to propose that all S-state transitions require Ca^{2+}. Here we confirm that interpretation by measurements of flash-induced S-state transitions in UV absorbance. The results are explained by a cation exchange at the Ca^{2+} binding site that, in the absence of the extrinsic PsbP and PsbQ polypeptides, can occur in minutes in low S-states and in seconds in high S-states, depending on the concentration of the substituting cation. In the $S_2(K^+)$ or $S_2(Na^+)$ state a slow conformational change occurs that prevents recovery of the slow-exchange situation on return to a lower S-state but does not inhibit the S-state cycle in the presence of Ca^{2+}. The ratio of binding affinities for monovalent vs divalent cations increases dramatically in the higher S-states. With the possible exception of S_0 to S_1, all S-state transitions specifically require Ca^{2+}, suggesting that Ca^{2+}-bound H_2O plays an essential role in a H^+ transfer network required for H^+-coupled electron transfer from the Mn cluster to tyrosine Z.

The Ca^{2+} ion in the oxygen-evolving complex (OEC)[1] of photosystem II (PSII) stabilizes Mn ligation (*1, 2*), and this probably plays an essential functional role during the assembly of the Mn_4Ca cluster that constitutes the active site (*3*). In addition, a variety of mono-, di-, and trivalent metal cations can act as competitive inhibitors of O_2 evolution (*4*), presumably by replacing Ca^{2+} at its binding site without assuming the essential functional role of the Ca^{2+} ion (e.g., refs *5–7*). 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 $Mn^V{=}O$ in S_4 to catalyze O−O bond formation (*8, 9*). However, those models do not explain why Ca^{2+} extraction inhibits O_2 evolution at other steps in the mechanism preceding O−O bond formation.

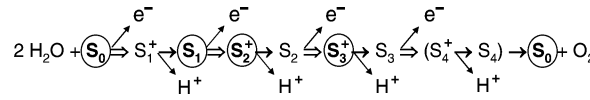
The O−O bond formation reaction takes place after the OEC has accumulated four oxidizing equivalents (*10*):

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

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

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

Nevertheless, the individual S-state transitions differ widely from one another in properties such as kinetics and temperature dependence. These differences can in part be rationalized by the need to restore electroneutrality to the PSII Mn cluster by H^+ release after each oxidation, in order to make the next oxidation of the complex thermodynamically feasible (*11*). The substrate water molecules may not be deprotonated until the last step, as indicated in the scheme above, but the 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 complex 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 highlighting the classical, semistable S-states, the cycle can be written as follows (*12*):



[†] This research was supported in part by a fellowship from the J. S. Guggenheim Foundation and a grant (MCB-0716541) to C.F.Y.

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[1] Abbreviations: Chl, chlorophyll (*a* + *b*); DCBQ, 2,6-dichloro-*p*-benzoquinone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; PsbP and PsbQ, 23 and 17 kDa extrinsic polypeptides of PSII; P_{680}, primary electron donor in the PSII reaction center; Q_A, first quinone electron acceptor in PSII; S-states, oxidation states of the OEC; $S_1(K^+)$, $S_2(K^+)$, S-states formed by Ca^{2+} displacement by K^+; $S_2(Na^+)$, the S_2 state formed by Ca^{2+} displacement by Na^+ in the light; Y_Z, tyrosine residue that acts as secondary electron donor in PSII.

10.1021/bi8006059 CCC: $40.75
Published on Web 07/02/2008

Unlike the oxidation of S_0, the oxidation of S_1 is not accompanied by spontaneous H^+ release. As a consequence, electron transfer to $Y_Z^\bullet$ on the subsequent flash will be rate limited by H^+ release, if the potential of S_2^+ is prohibitively high. 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 $P_{680}Y_Z^\bullet S_2$. If this is actually $P_{680}Y_Z^\bullet S_2^+$ and cannot proceed without H^+ release, its specific Ca^{2+} dependence might indicate that the H^+ comes from a Ca^{2+}-bound H_2O molecule. If so, the S_3 to S_0 transition could be Ca^{2+} dependent for the same reason, while no specific Ca^{2+} requirement might be expected for the S_1 to S_2 transition, which does not involve H^+ release.

Since the S_3 state cannot form in the absence of Ca^{2+}, there is at present no evidence for a possible Ca^{2+} requirement for the S_3 to S_0 transition. Most of the relevant literature proposes that Ca^{2+} is not required for the S_1 to S_2 transition, but we recently concluded (*13*) that the evidence 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. (*14*), who recently reemphasized the point that Ca^{2+} does not play an essential functional role in the S_1 to S_2 transition. The former explanation applies to preparations that have been treated to extract Ca^{2+} along with the extrinsic PsbP and PsbQ subunits by the most commonly used method, incubation of isolated PSII samples in 1−2 M NaCl.

Calcium depletion by NaCl treatment requires illumination of the sample (*15, 16*), because it is most effective in high S-states (*17*). The method produces samples that require Ca^{2+} addition for O_2 evolution activity under steady-state illumination but which nevertheless contain Ca^{2+} (*18*) unless special precautions are taken to prevent the residual Ca^{2+} contamination in the sample from rebinding after the treatment. Miyao and Murata (*16*) showed that NaCl treatment, in addition to removing the extrinsic PsbP and PsbQ polypeptides, converts the Ca^{2+} binding site to a state that facilitates rapid exchange of the metal. Therefore, loss of activity in a steady-state assay could occur due to loss of Ca^{2+} from the higher S-states if the Ca^{2+} binding affinity is decreased (*18*).

In order to further characterize the relationship between the S-state dependence of the Ca^{2+} requirement and its binding affinity, and the Ca^{2+} exchange rate, we examined the effects of Ca^{2+} depletion and reconstitution on the S-state transitions induced by a series of saturating flashes by monitoring the UV absorbance changes associated with these transitions.

MATERIALS AND METHODS

Photosystem II membranes were prepared from spinach according to Berthold et al. (*19*) with modifications (*20*) 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 treated with Chelex 100 to remove residual Ca^{2+}. For Ca^{2+} depletion and removal of the PsbP and PsbQ subunits at high ionic strength (*21, 22*), PSII membranes were suspended at 0.4 mg of Chl/mL in buffer A. After stirring for 5 min, an equal volume of 4 M NaCl or 2 M KCl along with 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added, giving final concentrations of 0.2 mg of Chl/mL, 2 M NaCl or 1 M KCl, and 1 mM EGTA. The mixture was incubated for 30 min in the dark and, where indicated, then exposed to room light for 30 min. To ensure homogeneous light exposure, the sample was spread in a thin layer on the flat bottom of a large Erlenmeyer flask. The salt-washed PSII membranes were centrifuged for 20 min at 48000*g* and resuspended in buffer A. Based on the approximately 100-fold dilution, residual concentrations of 20 mM Na^+ or 10 mM K^+ and 10 μM EGTA are expected. Extraction of the PsbP and PsbQ extrinsic polypeptides without Ca^{2+} depletion was done by a 15 min incubation of intact PSII membranes in 50 mM Na_2SO_4 and 50 mM HEPES, pH 7.5 (*23, 24*), followed by centrifugation and resuspension in buffer A. In most cases, salt-washed or pH 7.5/sulfate-treated samples were used directly, with a minimum delay between resuspension and the first measurement of about 15 min, but no obvious differences were observed if the preparations were stored at −80 °C before use. 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) plus 400 μM ferricyanide or 100 μM PPBQ (phenyl-*p*-benzoquinone) were added as indicated in the figure legends.

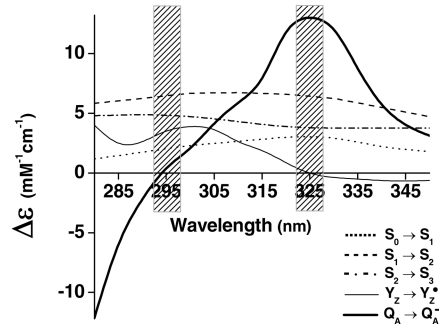


FIGURE 1: Absorbance difference spectra of S-state transitions, Y_Z oxidation, and Q_A reduction. Gray bars show the half-width of the spectral profile of the measuring light used here, which was centered at either 295 or at 325 nm to minimize Q_A or Y_Z contributions, respectively. Based on data from van Leeuwen et al. (*25*) and Dekker et al. (*26*).

Flash-induced S-state transitions were measured by UV absorbance changes with a single beam absorbance difference spectrophotometer. 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 ($Y_Z \rightarrow Y_Z^\bullet$) (Figure 1, thin line). This absorbance change is normally short-lived and seen mainly during the 2 ms $S_3 \rightarrow S_0$ transition, but its amplitude and kinetics will be affected

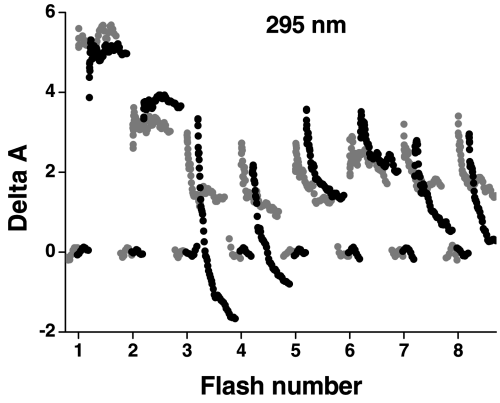


FIGURE 2: S-state absorbance changes in NaCl/EGTA-treated PSII. Photosystem II membranes were incubated in 2 M NaCl/1 mM EGTA for 30 min in the dark (gray traces, measured in the incubation medium) or for 30 min in the dark followed by 30 min in room light and resuspension in low salt medium without EGTA (black traces, measured in buffer A). The electron acceptor was 100 μM PPBQ. Flashes were spaced at 250 ms, and absorbance was measured at 295 nm from 20 ms before to 50 ms after each flash. For clarity, the black traces are shown at a slight *x*-offset.

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 (*15*). This reaction contributes only slightly at 295 nm (*27*) and is detected most easily by the decay of Q_A^- absorption at its 325 nm peak (Figure 1, heavy line). Flash-induced 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 (bars in Figure 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). Absorbance change (ΔA) amplitudes are indicated in absorbance units (mM PSII)$^{-1}$ cm^{-1} assuming 250 Chl/PSII, which was 10^4 times the actually measured value, corrected for flattening (*28*), and corresponds to $\Delta\varepsilon$ units in Figure 1. Fits to the Kok model (*10*) 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.

RESULTS

In order to determine the effects of a standard Ca^{2+} depletion treatment on S-state cycling, intact PSII membranes were treated with 2 M NaCl and 1 mM EGTA for 30 min in the dark and 30 min in room light, then centrifuged, and resuspended in buffer A. Figure 2 shows the absorbance changes induced by a series of saturating flashes in this preparation, measured at 295 nm where contributions by the electron acceptor side of PSII are small and the flash-induced changes are largely due to the S-state cycle. Although O_2 evolution measured with a Clark electrode depends on Ca^{2+} addition after such treatment (data not shown), the characteristic period 4 oscillation with flash number of S-state absorbance changes is not suppressed (black traces). The result shown here differs from an untreated control only in an increased miss probability and a much slower S_3 to S_0 transition (9 ms), effects that result from the high ionic strength treatment and are not repaired by the addition of Ca^{2+}. This result confirms that Ca^{2+} is bound to PSII after exposure to high ionic strength and a chelator (*18*) and shows that it is functional in supporting the full S-state cycle. However, when samples were taken from the NaCl treatment, after 30 min incubation in the dark in 2 M NaCl/1 mM EGTA but before illumination, and their flash-induced S-state absorbance changes measured in that mixture, the first flash amplitude was unaffected, but a strong suppression of the S-state cycle was observed after the first two flashes (Figure 2, gray traces). This result is remarkable, because inhibition of the S-state cycle by Ca^{2+} loss in the high S-states within seconds was predicted to occur (*13*), but only if PSII had been exposed to room light for tens of minutes during NaCl treatment (*16*).

To determine if Ca^{2+} displacement from its binding site could be enhanced, KCl was substituted for NaCl during exposure of PSII to high ionic strength (*5, 29*). Very different results were obtained, as shown in Figure 3. After exposure of PSII membranes to 1 M KCl/1 mM EGTA for 30 min at 4 °C 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 (Figure 3, left frames) the pattern after Ca^{2+} addition is normal, apart from the known effects of removing the extrinsic PsbP and PsbQ subunits by high ionic strength treatment. That is, 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 (data not shown)). 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.

In the absence of Ca^{2+}, however, the oscillation of absorption changes is suppressed (Figure 3, black circles). The pattern of absorbance changes is equivalent to that observed after inactivation of PSII by Tris treatment: Q_A^- is produced by the first flash 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 $Y_Z^{\bullet}$ 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 $Y_Z^{\bullet}$ 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 complete suppression of the S-state absorbance oscillation at 295 nm (Figure 3, lower left frame). At 325 nm a pronounced 0.3 ms $P_{680}^+Q_A^-$ recombination reaction is observed on all flashes including the first, and only a minor fraction of long-lived Q_A^- is detected. An expanded view of the first flash trace is shown in Figure 3B to illustrate the

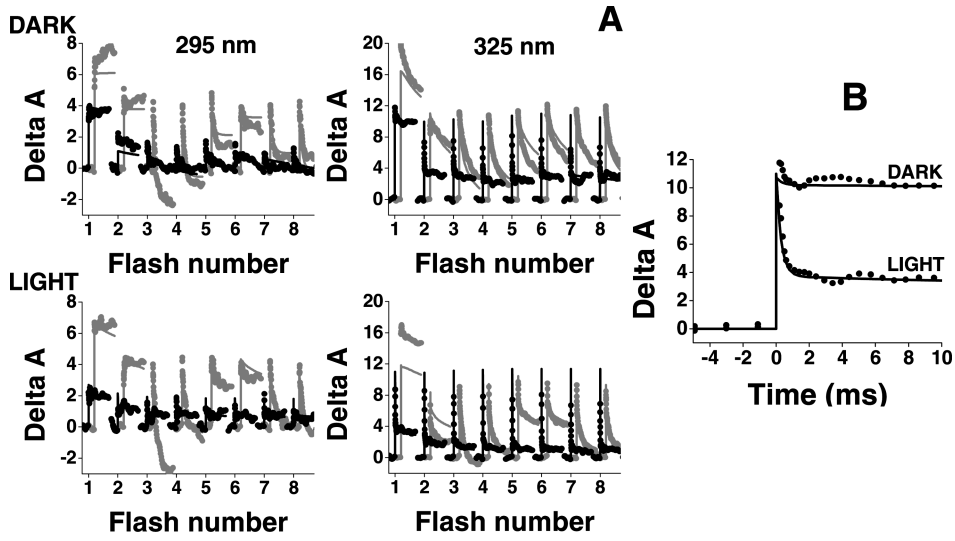


FIGURE 3: Effect on flash-induced absorbance changes at 295 and 325 nm of Ca^{2+} depletion using a KCl/EGTA treatment. Photosystem II membranes were depleted of Ca^{2+} by incubation in 1 M KCl/1 mM EGTA for either 30 min in the dark (upper frames) or for 30 min in darkness followed by 30 min in room light (lower frames), centrifuged, and resuspended in buffer A. Black circles: Absorbance changes in the absence of Ca^{2+}. Gray circles: 15 mM $CaCl_2$ added before measurements. The electron acceptors were 100 μM DCBQ and 400 μM ferricyanide. The time constant of the fast phase at 325 nm was 0.3 ms. The solid lines represent fits to the Kok model. Panel B: Expanded view of the first flash at 325 nm in room light exposed and dark incubated PSII samples depleted of Ca^{2+} by the KCl/EGTA treatment.

difference with the first flash response of the nonilluminated sample. This behavior persisted for at least 5 h in samples stored in darkness at 4 °C and may correspond to the "stable, modified S_2 state" (*30–32*). After addition of 15 mM $CaCl_2$ to such a sample, the absorption decrease associated with the S_3 to S_0 transition first occurred on the third flash (Figure 3A, gray circles), as in the nonilluminated sample, indicating that the illuminated sample was now in the S_1 state at the start of the flash series, as would be expected in view of the short lifetime of the higher S-states in these preparations in the presence of Ca^{2+}.

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 (which yielded a final $CaCl_2$ concentration of 70 mM) and the start of the flash series. Typical results from such an experiment are shown in Figure 4 (black circles). The data show that the absorption decrease at 295 nm on the S_3 to S_0 transition first occurred on the second instead of the third flash if the preparation had been exposed to room light during KCl/EGTA treatment (Figure 4, lower panel). This result confirms that PSII was in a S_2 state at the moment of Ca^{2+} addition. When the delay time between $CaCl_2$ addition and the first flash was extended to 10 s, a major fraction of centers had already decayed to S_1 (gray circles). The lines in Figure 4 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

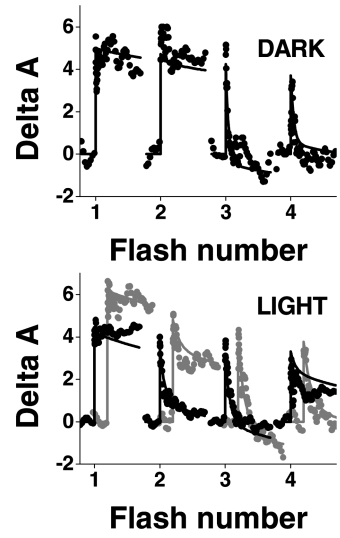


FIGURE 4: Effect of 70 mM $CaCl_2$ added immediately (2−3 s) before a flash series on absorbance changes at 295 nm to probe the S-state distribution after Ca^{2+} depletion as in Figure 3. Conditions are given in Figure 3. Upper frame: KCl/EGTA-treated in the dark (30 min). Lower frame: samples incubated with KCl/EGTA in darkness (30 min) and then in room light (30 min). Gray traces: Identical conditions, but with a 10 s delay between $CaCl_2$ addition and initiation of the flash series. The solid lines represent a global fit to the Kok model (see text).

S_2 state by the room light exposure during KCl/EGTA treatment was probably much larger than 54%.

The results presented in Figure 5 show that Ca^{2+} depletion could be obtained by treatment with 1 M KCl alone, without addition of EGTA to remove the Ca^{2+} that is nonspecifically bound to the PSII membranes. 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

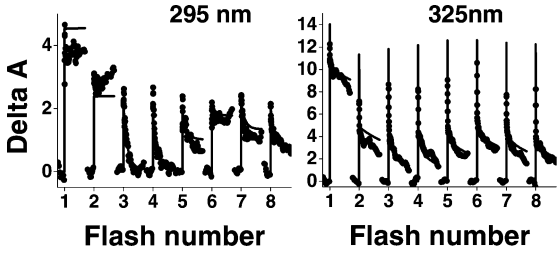
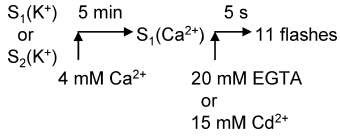


FIGURE 5: Effect of EGTA omission during KCl treatment on flash-induced absorbance changes at 295 and 325 nm. The experimental conditions are identical to those given in Figure 3, lower frames, except that EGTA was omitted during Ca^{2+} extraction from PSII by exposure to 1 M KCl in a 30 min dark, 30 min room light incubation. Solid lines: Fit to the Kok model, showing an initial distribution of 25% active centers, 52% centers in $S_1(K^+)$, and 23% centers in $S_2(K^+)$.

the Ca^{2+}-free PSII was in the S_1 state rather than S_2. Presumably the residual Ca^{2+} destabilized the $S_2(K^+)$ state but was not sufficient to prevent K^+ rebinding after decay to the $S_1(Ca^{2+})$ state had occurred. In view of the ~10 s lifetime of $S_2(Ca^{2+})$, a 1% population of that state would be expected to cause a decay to S_1 in the 15 min between sample resuspension after centrifugation and the beginning of measurements.

In order to determine the effect of room light exposure used to extract Ca^{2+} in the presence of KCl and EGTA on the accessibility of the Ca^{2+} site in PSII, a series of experiments were carried out using the strategy represented diagrammatically as follows:



Calcium-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 Figure 4. At least 5 min 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 gray traces in Figure 6 (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 6, when a Ca^{2+} chelator (20 mM EGTA, upper frames) or a competitive inhibitor of Ca^{2+} binding (15 mM Cd^{2+}, lower frames) was added 5 s 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. The pattern after Cd^{2+} addition suggests that all centers in S_1 were 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 (*16*) that illumination during exposure of PSII to high ionic strength changes PSII to an "open" conformation where OEC Ca^{2+} 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

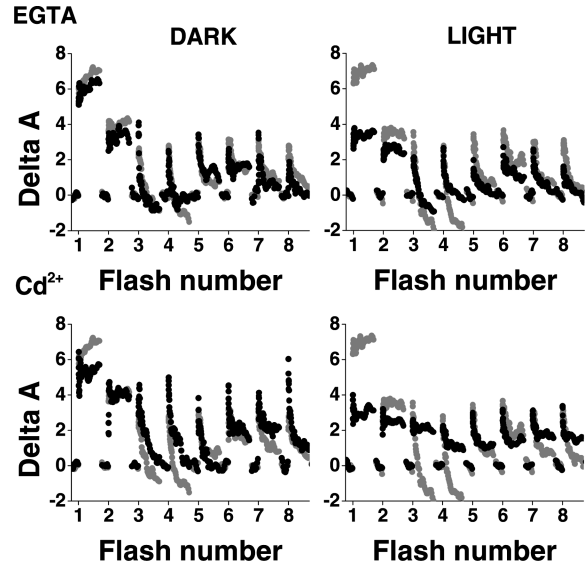


FIGURE 6: Effect of room light exposure during KCl/EGTA treatment on accessibility of the Ca^{2+} site in the S_1 state. Photosystem II membranes were Ca^{2+}-depleted as in Figure 3 by exposure to 1 M KCl/1 mM EGTA either for 30 min in the dark (left frames) or for 30 min in dark and 30 min in room light (right frames) and resuspended in buffer A. All samples were converted to the active $S_1(Ca^{2+})$ state by incubation with 4 mM $CaCl_2$ for at least 5 min before use. Black traces: 20 mM EGTA (upper frames) or 15 mM Cd^{2+} (lower frames) was added 5 s before measurement of flash-induced absorbance changes at 295 nm. Gray traces: No addition (same data in upper and lower frames). The electron acceptor was 100 μM PPBQ in all experiments.

treatment, provided that the extrinsic PsbP and PsbQ polypeptides had been removed. This can be achieved by a 15 min exposure to pH 7.5/50 mM Na_2SO_4 (*23, 24*), a treatment that avoids both illumination and high ionic strength, does not extract Ca^{2+}, and yields preparations that are fully active if Cl^- is present in the medium. Figure 7 presents the results of experiments using this preparation. In order to avoid possible complications by irreversible effects of Cd^{2+} addition, samples were exposed to 1 mM $CdCl_2$ for 15 min, followed by washing with 3 mM EGTA to remove the Cd^{2+}. This treatment did not remove Ca^{2+} from its binding site. In the presence of 15 mM $CaCl_2$, flash-induced absorption changes were measured as in Figure 6, but the flash series was interrupted briefly for Cd^{2+} addition before flash 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, respectively. The traces of the first four flashes after Cd^{2+} addition are shown. When added in the S_2 or S_3 state (panels 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 s after addition. In low S-states Cd^{2+} had no effect in 5 s (not shown), and even after 30 s, as shown in panels A and B, there was little difference in comparison to the control (no addition, shown in gray). The minor effects seen in panel B are largely due to inactivation of centers that were still in higher S-states after four 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 four 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 lack of inhibition in low S-states was not due to a low affinity; complete inhibition was observed at longer incubation times,

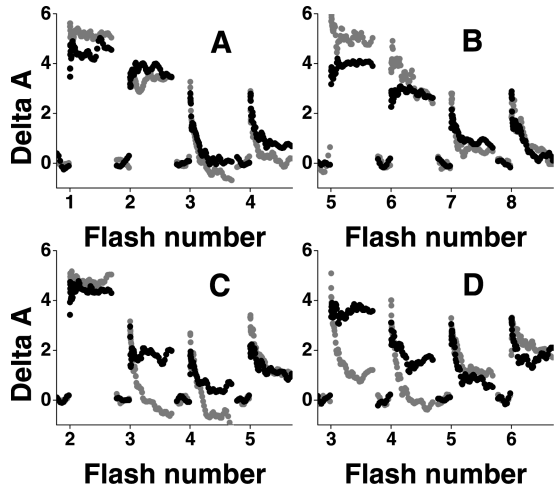


FIGURE 7: S-state dependence of Cd^{2+} accessibility and substitution for Ca^{2+}. Photosystem II membranes were depleted of PsbP and PsbQ by pH 7.5/sulfate treatment, which leaves Ca^{2+} bound to its site, and resuspended in buffer A. Absorbance changes were measured at 295 nm in the presence of 15 mM $CaCl_2$ using 100 μM DCBQ as the electron acceptor. The flash series was interrupted for addition of 5 mM $CdCl_2$ before flash numbers 1 (panel A), 2 (panel D), 3 (panel D), or 5 (panel B). The flash train resumed after 30 s (A and B, low S-states) or 5 s (C and D, S_2 and S_3, respectively). The first four flashes after $CdCl_2$ addition are shown, as indicated by the numbers on the abscissas of the panels. Gray traces: The same measurements without Cd^{2+} addition.

inversely proportional to the Cd^{2+} concentration added. The fast exchange in high S-states is consistent with the inhibition by 2 M NaCl/1 mM EGTA after dissociation of PsbP and PsbQ from PSII observed in Figure 2, gray traces.

The S-state dependence of the ratio of the K^+ and Ca^{2+} binding affinities was investigated in PSII membranes that had been depleted of PsbP and PsbQ by pH 7.5/sulfate treatment and then Ca^{2+} depleted by KCl/EGTA treatment in the dark. These samples did not exhibit 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 gray traces in Figure 8A. This indicates 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 upon sample resuspension after KCl treatment and centrifugation would yield an estimated 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 preceding the flash train, 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 Figure 8A 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 9).

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 s. This is much shorter than the lifetimes of S_2 and S_3 (34 and 18 s, respectively) in this preparation. If K^+ was added after one flash, the S_3 to S_0 absorption transient on flash number 3

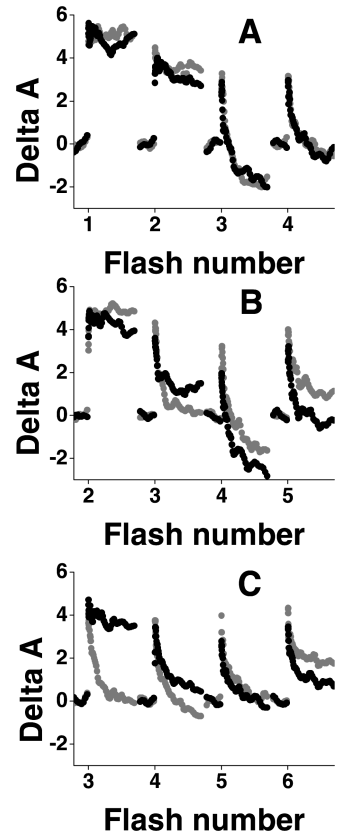


FIGURE 8: S-state dependence of the binding competition between K^+ and Ca^{2+}. Photosystem II membranes were depleted sequentially of PsbP and PsbQ by pH 7.5/sulfate treatment,and Ca^{2+} by KCl/EGTA treatment in the dark, centrifuged, and resuspended in buffer A. Samples were first reactivated by incubation with $CaCl_2$; KCl was added 5 min before flash number 1 (panel A) or 5 s before flash number 2 (panel B) or 3 (panel C) in order to determine at which $[K^+]/[Ca^{2+}]$ concentration ratio K^+ 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 number 3. The first four flashes after KCl addition are shown. The $CaCl_2$ and KCl additions were as follows: panel A, 2 and 100 mM; panel B, 10 and 250 mM; panel C, 15 and 50 mM. Gray traces: The same experiments without KCl addition. The electron acceptors were 100 μM DCBQ and 400 μM ferricyanide in all experiments.

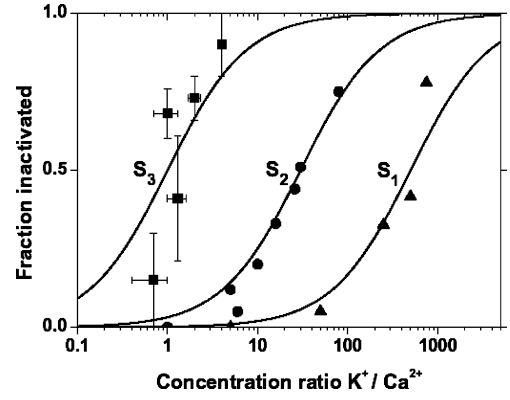


FIGURE 9: Relationship between extent of inhibition and the $[K^+]/[Ca^{2+}]$ ratio. The data are taken from experiments carried out as shown in Figure 8. The lines are fits assuming a binding competition at a single site with an S-state-dependent K_d ratio, yielding values of 500, 30, and 1 for S_1, S_2, and S_3, respectively. See the text for an explanation of the error bars in the case of S_3.

was nearly halved at a K^+/Ca^{2+} concentration ratio of 25 (Figure 8B). The transient was almost abolished (Figure 8C) after two flashes at a ratio of 4 (taking the residual 10 mM

K^+ into account). It was not possible to demonstrate any inhibition in S_0, but this involves measurements of the second S_3 to S_0 transition in the flash series, on flash numbers 7 and 8 (as in the experiment shown in Figure 7B), 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 9), indicates apparent K_d ratios (based on the concentrations added) of 500, 30, and 1 in the S_1, S_2, and S_3 state, respectively. The apparent K_d ratio in the S_0 state may be higher than 500. Somewhat erratic results were obtained for S_3. This anomaly cannot be explained by the uncertainty in the residual K^+ concentration and its effect on S_3 in the control data, because an error in the 10 mM estimate for residual K^+ would change all points in the same direction. The error bars shown in Figure 9 indicate the effect that ± 5 mM K^+ would have. An error due to incomplete equilibration in 5 s could be substantial, 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 competitive inhibitor Cd^{2+}, for which similar experiments indicated half-inhibition at Cd^{2+}/Ca^{2+} concentration ratios of about 0.2 and 0.1 in S_1 and S_2, respectively (data not shown).

A remarkable feature of the measurements where Cd^{2+} (Figure 7C) or K^+ (Figures 8B and 9) was added to a sample in the S_2 state, after one 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 oxidation of Y_Z to $Y_Z^{\bullet}$ at 295 nm. However, the Ca^{2+}-free $S_2(K^+)$ state, in contrast to the $S_1(K^+)$ state, was shown in Figure 3 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 requires more time than the few seconds available in these experiments.

In the case of Cd^{2+} addition after the first flash (Figure 7C), however, the absence of charge recombination on the second flash has a different explanation. Cadmium failed to decrease the flash yield of $Y_Z^{\bullet}$ formation in any S-state. As shown in Figure 10, 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, panel A) could be suppressed completely by substituting Cd^{2+} for K^+ (panel 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 (panel C). Since the $S_1(K^+)$ state does not exhibit the 0.3 ms transient on the first flash (Figure 3), the sample was still in the S_2 state.

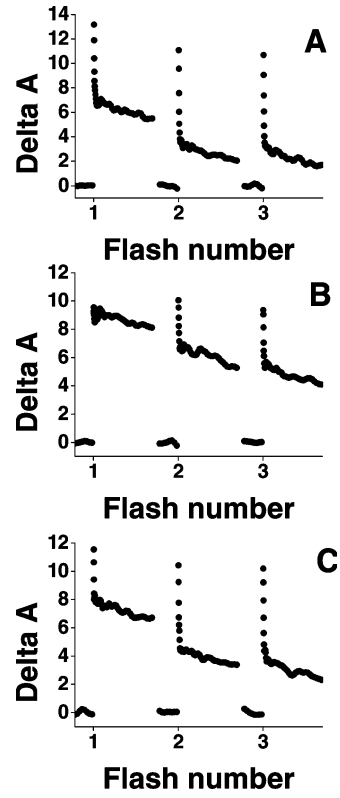


FIGURE 10: Reversible suppression of $P_{680}^+Q_A^-$ recombination by substitution of Cd^{2+} for K^+ in the stable, modified $S_2(K^+)$ state, measured at 325 nm on the first flash. Photosystem II membranes were Ca^{2+}-depleted by treatment with 1 M KCl/1 mM EGTA (30 min dark incubation followed by 30 min room light exposure) and resuspended in buffer A. The electron acceptors were 100 μM DCBQ and 400 μM ferricyanide. Panels: A, no addition; 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.

DISCUSSION

These results confirm that K^+ is more effective than Na^+ in removing Ca^{2+} from its functional site in PSII (*5, 33*) and suggest that, after dissociation of PsbP and PsbQ by high ionic strength, these cations can replace Ca^{2+} in a binding competition (*34*) where K^+ shows higher affinity for the site than Na^+ (*5*). In the dark, even 2 M Na^+ is unable to replace Ca^{2+}, which explains why trapping the $S_2(Na^+)$ state produced in the light requires either rebinding of PsbP and PsbQ (*30*) or addition of a Ca^{2+} chelator immediately after illumination at high ionic strength (*29*). No such precautions are required to stabilize the corresponding $S_2(K^+)$ state. In the case of KCl treatment in the dark, 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 data in Figure 5 show that accumulation of the stable $S_2(K^+)$ state requires more rigorous Ca^{2+} depletion than $S_1(K^+)$, which implies that formation of the stable state occurs only when Ca^{2+} has been replaced by K^+ in the S_1 state. Apparently, stable $S_2(K^+)$ is 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, indicating that the conversion of $Y_Z^{\bullet}S_1(K^+)$ to $Y_ZS_2(K^+)$ is slow in comparison to its lifetime of a few seconds. However, this conversion is

complete within 30 min under continuous illumination, which probably maintains Y_Z in the oxidized state in a significant fraction of the centers. A time constant between 10 s and 10 min would indicate that the oxidation of S_1 to S_2 by $Y_Z^{\bullet}$ is 5−7 orders of magnitude slower than the 0.1 ms observed in the presence of Ca^{2+} (*35*). This would inhibit water oxidation 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 redox 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+}.

Ca^{2+} Accessibility and Affinity. Current models of the Mn_4Ca cluster (*36*) are consistent with the observation that high concentrations of competing metals or low pH can remove Ca^{2+} from its site and that 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 here to be more than 1 h 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 fluctuations and inherent properties of the binding site. If 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, such a rate limitation 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 (*18*). This might be explained by the H^+ concentration if ligand protonation is required, because Ca^{2+} is known to be released from PSII within 5 min at pH 3 (*37*).

The time required for cation substitution at the Ca^{2+} 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 or 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+} (*38*), which is reversed when the cycle returns to a low S-state. 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 (Figure 7) and the drastic change in the relative affinities of the binding site for monovalent and divalent cations (Figure 9). 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. (*6*). This allows binding of K^+ in the S_1 state, presumably because K^+ with an ∼1.4 Å ionic radius is larger than either Na^+ or Ca^{2+}, whose ionic radii are about 1 Å. However, K^+ exhibits a 500 times lower apparent affinity than Ca^{2+} for the S_1 state, while in the S_3 state the apparent 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 Ca^{2+} ions, but not K^+ ions, may be bound nonspecifically to the membranes (*39, 40*), 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+} should be much higher than that of the PSII Ca^{2+} binding site. Inhibition of the S-state cycle by EGTA could be explained by a decrease in the external Ca^{2+} concentration (essentially to zero if more EGTA than Ca^{2+} was present) relative to that of any competitive inhibitor (or H^+) present in the sample; the rate of inhibition was independent of the EGTA concentration. Also, the instability of the Ca^{2+}-free S_2 state after KCl/light treatment without EGTA (Figure 5) is fully accounted for by the increased Ca^{2+} contamination of the sample: At a Ca^{2+}/K^+ concentration ratio that would leave one-third of S_1 active, as estimated by the fit in Figure 5, the binding curves in Figure 9 predict that 3% of the S_2 state would have Ca^{2+} bound, which would cause an S_2 decay time of 5 min (100/3 times the 10 s lifetime of $S_2(Ca^{2+})$ in KCl-treated PSII). Since the residual K^+ concentration is estimated at 10 mM, the S_1 binding curve in Figure 9 indicates that the residual Ca^{2+} contamination in the sample of Figure 5 was near 10 μM. The destabilization of $S_2(Na^+)$ by the same Ca^{2+} contamination is likely to be even faster and to result in a quantitative conversion to the active $S_1(Ca^{2+})$ state. The apparent dependence of the stable S_2 state on the use of chelators during Ca^{2+} depletion has led to proposals that its properties result from chelator binding to the Mn cluster. We found no need to postulate that here or in the literature (*13*). Moreover, in view of the binding competition between acetate and chloride in the OEC with a K_d ratio of 30 (*41*), binding of a chelator oxoanion ligand at 1 mM EGTA in the presence of 1−2 M Cl^- is quite unlikely, and the concentration ratio is even lower after KCl/EGTA treatment in the present work (10 μM EGTA and 40 mM Cl^-).

Since EGTA 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 the chelator had no effect in the presence of 2 M Na^+ (Figure 2), which demonstrates that Na^+ cannot bind in S_1. In the "open conformation" EGTA caused half-inhibition in 5 s in a sample where the only potential competitive inhibitor was the estimated 10 mM K^+ remaining after KCl treatment (Figure 6). Apparently, K^+ can replace 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 ionic strength under room light. This is about 10^3 times faster than in samples that had not been exposed to room light, such as in the S_1 experiments of Figure 9 where equilibration took a few minutes (an equilibration rate constant of 0.02 s^{-1} in the 2 mM Ca^{2+}/1 M K^+ mixture, where the Ca^{2+} and K^+ binding rates are equal, would mean a K^+ binding rate constant of 0.01 M^{-1} s^{-1}). 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.

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 Ca^{2+} carboxylate ligand, is a normal property of intact PSII. This would be of little or no consequence in the presence of PsbP and PsbQ, but in their absence Ca^{2+} may be replaced by a monovalent cation (K^+, Na^+). The long lifetime of the trapped S_2 state allows a secondary structural rearrangement to take place, possibly a movement of the amino acid providing the carboxylate ligand, which prevents rapid recovery of the native structure when Ca^{2+} rebinds and the OEC returns to the S_1 state. The data of Miyao and Murata (*16*) suggest that the rearrangement takes 20 min and its reversal in the presence of Ca^{2+} takes about 1 h. In the experiments of Boussac and Rutherford (*17*) the rearrangement was largely complete in 10 min if all centers were in a high S-state.

This modification 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 likely to be necessary to avoid such inhibition. A similar conclusion was drawn earlier for the essential Cl^- ion in the OEC (*42*).

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 by 70−80%, similar to the finding reported by Andréasson et al. (*43*) 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. The data presented here provide 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 (Figure 3) or by the pH 3/citrate treatment of Ono and Inoue (*37*) (not shown). However, Haumann and Junge (*44*) did observe a similar low flash yield of Y_Z oxidation in pH 3/citrate-treated PSII core particles. The effect was much larger than can be explained by the slower $Y_Z^\bullet$ 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 experiments reported here show that the effect could be reversibly suppressed by replacing K^+ with Cd^{2+} (Figure 10), it is probably due to the charge deficit in the Ca^{2+} site but is not associated with the essential function of Ca^{2+} in $Y_Z^\bullet$ 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 10 also shows that the extreme stability of the Ca^{2+}-free S_2 state 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+}. Since Ca^{2+} binding restores these reactions immediately, but requires 1 h to convert the open conformation of its binding site back to the normal state (*16*), the stability of the Ca^{2+}-depleted S_2 state is probably also instantaneous and not induced by the conformational change.

Ca^{2+} Function. The data presented here confirm our interpretation of the literature on Ca^{2+} depletion proposed in ref *13* and show that the specific requirement for Ca^{2+} is in electron transfer from the Mn cluster to $Y_Z^\bullet$ on each of the S-state transitions, with the possible exception of the S_0 to S_1 transition, where conclusive evidence has yet to be obtained. The Ca^{2+} ion is redox-inert and Cd^{2+}, with the same charge and size, is inhibitory. The cation specificity of the Ca^{2+} requirement suggests stringent limits on the K_d for dissociation of a proton from a cation-ligated H_2O (*6*). Reduction of $Y_Z^\bullet$ requires protonation, and oxidation of the Mn cluster may require deprotonation of a ligand (e.g., ref *45*). If Ca^{2+} is situated between Y_Z and Mn (*46–48*), an 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, Ca^{2+}-bound H_2O might be involved in O−O bond formation on the S_3 to S_0 transition (*8, 9*). The data presented here demonstrate a specific Ca^{2+} requirement for O_2 evolution in the S_1 to S_2 and S_2 to S_3 transitions as well. The Ca^{2+} requirement for the S_1 to S_2 transition, which is not accompanied by H^+ release from PSII, may support the view that $Y_Z^\bullet$ reduction is coupled to Ca^{2+}-mediated proton transfer from the Mn cluster and therefore does not rebind the proton from the base that is protonated during Y_Z oxidation, as proposed by Dau and Haumann (*11*). Styring et al. (*49*) 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 $Y_Z^\bullet$, rather than its failure to deprotonate an Mn ligand. This is not easily explained if Y_Z oxidation in the same system is inhibited because its normal proton acceptor is already protonated, as concluded by Haumann and Junge (*44*). It is tempting to speculate that, somehow, the proton donor for $Y_Z^\bullet$ reduction cannot be the base that acts as the proton acceptor during Y_Z oxidation, so that the system actually functions as a proton pump.

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BI8006059