The Photosynthetic Oxygen Evolving Complex Requires Chloride for Its Redox State $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ Transitions But Not for $S_0 \rightarrow S_1$ or $S_1 \rightarrow S_2$ Transitions†

Hanna Wincencjusz,‡ Hans J. van Gorkom,‡ and Charles F. Yocum*,§

Biophysics Department, Huygens Laboratory, State University, P.O. Box 9504, NL-2300 RA Leiden, The Netherlands, and Departments of Biology and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-1048

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ABSTRACT: The Cl^- requirement in the redox cycle of the oxygen-evolving complex (OEC) was determined by measurements of flash-induced UV absorbance changes in Cl^--depleted and Cl^--reconstituted photosystem II membranes. On the first flash after dark adaptation the spectrum and amplitude of those changes, known to reflect the oxidation of Mn^{III} to Mn^{IV} on the $S_1 \rightarrow S_2$ transition, were the same in the presence or absence of Cl^-. On the second and later flashes, however, absorbance changes in Cl^--depleted samples revealed only electron transfer from tyrosine to quinone which reversed slowly in the dark by charge recombination and did not produce the S_3-state. A rapid method was developed to remove Cl^- after producing the S_3-state by two flashes. The lifetime of the S_3-state was found to be unaffected by Cl^--depletion, in contrast to the 20-fold stabilization of the S_2 lifetime by Cl^- removal, and the Cl^--depleted S_3-state did not proceed to S_0 on flash illumination. However, when the same Cl^--depletion procedure was applied after producing the S_0-state by three flashes, further advance to S_2 by two additional flashes was not impaired by the absence of Cl^-. The requirement for Cl^- only on the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions can be rationalized by the hypothesis that Cl^- is required for electron transfer between manganese ions within the oxygen-evolving complex.

Oxygenic photosynthesis uses water as the ultimate source of electrons. To this end, the oxygen-evolving complex (OEC)[1] of Photosystem II (PS II) couples a one-electron reaction, photochemical charge separation, to the four-electron oxidation of water. The four-step accumulation of oxidizing equivalents and their subsequent release in water oxidation are described by the S-state cycle (Kok et al., 1970). The OEC contains four manganese atoms that are widely considered to be the site of the redox reactions leading to water oxidation. However, Ca^{2+} and Cl^- are also required for productive S-state advancements that culminate in water oxidation (Debus, 1992; Yocum, 1991). The role of Cl^- in the OEC is not well established, and the site at which the ion binds is likewise not known with certainty, although it must be in close proximity to the manganese cluster. Sandusky and Yocum (1984) proposed that Cl^- acts as a bridging ligand between manganese ions in the OEC to facilitate electron transfer within the metal cluster. The suggestion that Cl^- is a Mn ligand was based on the fact that Cl^- and primary amines compete for a common binding site in the OEC under steady-state conditions. The effectiveness of amines in this competition was proportional to their basicity, suggesting that they bind as Lewis bases to a Lewis acid, in this case Mn in an oxidation state $>+2$. Alternatively, Cl^- binding close to the active site of water oxidation was proposed to neutralize and thus to stabilize the accumulated positive charges on the S-states (Govindjee et al., 1983). A protective role in which the anion prevents premature discharge of oxidizing equivalents stored in the OEC has been postulated recently (Hoganson et al., 1995; Babcock, 1995). In this model, the halide was proposed to bind to one manganese atom and only in the S_0 and S_1-states.

Lindberg and Andréasson (1993) utilized $^{36}Cl^-$ to show that a single atom of Cl^- is bound to the OEC. The kinetics of Cl^- release demonstrate that in S_1 the anion exchanges slowly with the external medium and is only displaced efficiently by other anions (Br^-, NO_3^-) that activate water oxidation. Removal of the extrinsic 23 and 17 kDa polypeptides, and also Cl^- depletion of the intact enzyme complex, modifies the behavior of the Cl^- site; binding now occurs with a lower affinity and the halide exchanges rapidly (Lindberg & Andreasson, 1995). Upon addition of Cl^-, the site reverts to its original (high-affinity, slow exchange) behavior.

S-state advancements in Cl^--depleted PS II have been probed by several techniques. Early fluorescence data reported storage of two oxidizing equivalents after removal of Cl^- (Itoh et al., 1984; Theg et al., 1984) and EPR data revealed an inhibition of S-state transitions after reaching an altered S_2-state (Ono et al., 1986b). Further spectroscopic investigations of the effects of Cl^- removal from the OEC have been concerned with consequences for formation of the S_2 multiline signal and for Mn oxidation reactions as

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* Address correspondence to this author at Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048. Phone: (313) 647-0897. FAX: (313) 647-0884. E-mail: cyocum@umich.edu.

‡ Huygens Laboratory.

§ The University of Michigan.

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; MES, 2-[*N*-morpholino]ethanesulfonic acid; OEC, O_2-evolving complex; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; SDS, sodium dodecyl sulfate; Tricine, *N*-tris[hydroxymethyl]methyl glycine; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].

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monitored by XANES edge shifts or H^+ release. The EPR results [reviewed by Rutherford (1992) and Britt (1996)] show that addition of F^- to the OEC to replace Cl^- results in formation of the $g = 4.1$ EPR signal rather than the $g = 2$ multiline signal. Under conditions of Cl^--depletion by SO_4^{2-} treatment, it is proposed that an organic radical is formed on the $S_1 \rightarrow S_2$ transition and that Mn oxidation then occurs on the $S_2 \rightarrow S_3$ transition to produce the "split S_3" signal (Boussac & Rutherford, 1994), attributed to an interaction of the S_2-state with an organic radical now identified as Y_Z^+ (Gilchrist et al., 1995). Under Cl^--depleted conditions, XANES edge shifts to higher energy are indicative of Mn oxidation arrested at S_2 (Ono et al., 1995), but it is also claimed from analyses of XANES edge energies that Mn oxidation, plus or minus Cl^-, does not occur beyond S_2 (Roelofs et al., 1995). Proton release is also blocked beyond S_2 by Cl^--depletion (Lübbers et al., 1993). Finally, a recent report by Haumann et al. (1995), presenting absorption difference spectra for the UV−vis region, suggested that illumination of dark-adapted, Cl^--depleted PS II does not lead to Mn oxidation. Instead, an oxidations of His and Tyr on the first and the second flashes, respectively, were proposed.

Better founded theories concerning the role of Cl^- in PS II would be possible if the Cl^--dependence of the individual S-state advancements were known. A report by Preston and Pace (1985) presents the only data so far on S-state dependent Cl^- binding to PS II. Using ^{35}Cl NMR the authors showed that centers in the S_2 and S_3-states bind Cl^- with an affinity comparable to that which activates oxygen evolution. In contrast no detectable high-affinity binding was found in the lower S-states. The latter result is contradicted by Lindberg et al. (1993) who showed that in the dark-adapted S_1-state PS II binds Cl^- with high affinity. It is possible that the high Cl^- affinity of the S_1-state prevents detection of the anion by the NMR technique used by Preston and Pace.

UV−vis spectroscopy allows direct observation of donor side events in Photosystem II. The measurements can be performed relatively quickly and Cl^--depletion/addition can be carried out directly in the measuring cuvette. We have used a rapid method of Cl^--depletion and measured UV absorbance changes induced by single-turnover flashes to probe the Cl^--requirement for each of the S-state transitions. The results reported here show that Cl^- is required only for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions.

MATERIALS AND METHODS

Photosystem II membranes (so-called BBY preparations) were isolated from spinach as described by Berthold et al. (1981) with the modifications described by Ghanotakis et al. (1984). For some preparations, Cl^- was omitted from the buffer used in the wash steps after Triton X-100 treatment. The PS II membranes prepared in this way with "Cl^--free" buffer did not exhibit any Cl^- deficiency when their activity was assayed at pH 6.0, either by the flash-number dependence of light-induced UV absorbance changes or by the rate of oxygen evolution measured polarographically in continuous light. Chloride depletion of dark-adapted PS II membranes was achieved by 15 min of incubation in a buffer containing 50 mM Hepes (pH 7.5), 0.3 M sucrose, and 50 mM Na_2SO_4 at a Chl concentration of 0.2 mg/mL. Measurements of UV−vis absorption changes were carried out immediately after incubation. The artificial electron acceptors DCBQ and ferricyanide (final concentrations of 50 and 100 μM, respectively) were added to avoid limitations from the PS II acceptor side.

In order to probe the Cl^--requirement of the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transition, a new procedure for rapid Cl^--depletion was developed. At pH 7.5 an incubation time of at least 3−5 min was required to remove Cl^-. This is substantially longer than the S_3 lifetime, which was determined to be less than a minute in intact PS II membranes (data not shown). This obstacle was overcome as follows: Cl^--depletion could be achieved in about 15 s by raising the sample pH to 8.5 in the presence of 50 mM Na_2SO_4. The procedure was performed directly in the measuring cuvette by injecting a mixture of concentrated Tricine (pH 8.5) and Na_2SO_4 to yield final concentrations of 50 mM for both reagents. For control measurements, where high Cl^- concentrations were maintained, the addition also contained NaCl to produce a final concentration of 0.2 M in the measuring cuvette.

Absorbance difference measurements were carried out at room temperature in a single-beam apparatus described previously, using a tungsten halogen lamp for wavelengths above 290 nm and a deuterium lamp below 290 nm (Dekker et al., 1984a). The optical path length was 1.2 mm. Saturating excitation flashes were generated by a Nd:YAG laser (532 nm, half-width 6 ns). For all measurements a flash frequency of 1 Hz was used and the instrument time constant was set at 0.5 ms. Each trace presented in the figures represents 50 ms unless otherwise stated. The photomultiplier was protected from the excitation flashes by appropriate filter combinations. The S-state advancements could be conveniently probed at 295 nm, where absorbance changes due to the acceptor side of PS II are minimal.

Steady-state rates of oxygen evolution were assayed with a Clark electrode at 20 °C in the presence of 2.5 mM ferricyanide and 0.4 mM DCBQ at a Chl concentration of 10 μg/mL. Gel electrophoresis was carried out as described by Chua (1980) using 13.5% acrylamide in the presence of 4 M urea. The residual Cl^- contamination of buffers was determined as described by Lindberg et al. (1993) and was routinely found to be in the range 20−40 μM Cl^-.

RESULTS

Sample Characterization. Incubation of intact PS II membranes at pH 7.5 in the presence of sulfate (Sandusky et al., 1983) proved to be an effective, quick, and convenient method for Cl^--depletion. Release of the halide could also be observed in the absence of sulfate at elevated pH, but under these conditions, the depletion process required hours (data not shown). The combination of SO_4^{2-} and pH 7.5 dissociates the 23 and 17 kDa extrinsic polypeptides (Homann, 1988). The effects of pH 7.5, Na_2SO_4 incubation on the polypeptide composition of PS II membrane preparations were analyzed by SDS−PAGE as shown in Figure 1. The release of 17 and 23 kDa proteins was observed, and this effect was maximal after 15 min, in agreement with the time required to deplete Cl^-. Moreover, dissociation of extrinsic proteins was almost independent of the presence of Cl^- in the suspending medium (see lanes 3 and 4 and lanes 5 and 6). Lanes 1 and 2 present typical results obtained with control and salt-washed PS II membranes, respectively.

Although the sulfate-treated PS II membranes appear to have the protein composition and Cl^--dependent activity of

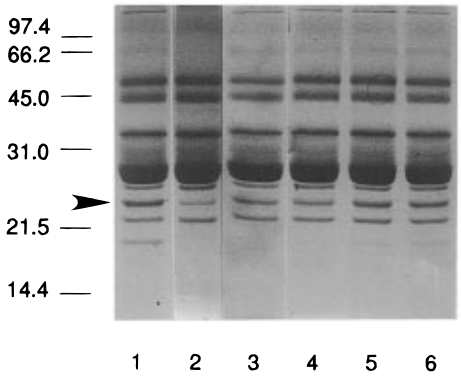


FIGURE 1: Effect of pH and SO_4^{2-} on the protein composition of PS II membranes. Numbers to the left of the gel indicate positions of molecular weight markers in kDa. The arrow indicates the 23 kDa extrinsic protein, which comigrates with an integral PS II protein under the conditions employed. Lane 1, control; 2, salt-washed; 3, incubated 1 h at pH 7.5 in the presence of 50 mM Na_2SO_4; 4, incubated 15 min as in 3; 5, incubated 1 h at pH 7.5 in the presence of 50 mM Na_2SO_4 and 50 mM NaCl; 6, incubated 15 min as in lane 5. The incubations at pH 7.5 were carried out at a Chl concentration of 0.2 mg/mL, on ice in darkness. After incubation, samples were collected by centrifugation and prepared for SDS−PAGE as described in Materials and Methods.

salt-washed PS II membranes (Ghanotakis et al., 1985), they retain Ca^{2+}; O_2-evolving activity in continuous light was only slightly dependent on addition of calcium ions [10−15% higher activity after addition of 20 mM calcium; this difference in activity explains the inhibitory action of sulfate reported by Sandusky and Yocum (1984)]. Of more importance to the present study, however, is the fact that in comparison to salt-washed PS II membranes, the pH 7.5/SO_4^{2-}-depleted preparations reconstituted with Cl^- produced a higher flash yield of S-state transitions (fewer "misses") resulting in more pronounced period four oscillations, little inactivation at pH 8.5, and slower deactivation of S_2 and S_3 (data not shown). The data presented in Figure 2 show that after incubation at pH 7.5 with 50 mM Na_2SO_4, the period four oscillation of UV absorbance changes induced by a series of saturating flashes was lost but could be fully restored by addition of 50 mM Cl^- (Figure 2A and 2B, respectively). With other methods of Cl^--depletion we could not obtain such an extensive, reversible suppression of period four oscillations, even when Clark electrode measurements indicated 100% inhibition. This latter observation is presumably due to both the greater sample dilution in Cl^--free assay medium (compared to that used for the optical measurements) and to the large number of turnovers required to detect oxygen evolution with a standard Clark electrode. Rates in control samples at pH 7.5, in the presence of saturating Cl^- and Ca^{2+}, were typically 350−450 μmol of O_2/h/mg of Chl and were stable for about 30 s. In the absence of Ca^{2+} the rates were lower by about 10−15%. In the absence of Cl^- no activity was observed. Instability of DCBQ at pH 8.5 interfered with comparable measurements of steady-state activity at this pH.

Cl^--Requirement of the $S_1 \rightarrow S_2$ Transition. The dark-adapted OEC is in the state S_1, and illumination by a single-turnover flash brings about the $S_1 \rightarrow S_2$ transition, which produces a well-characterized UV-absorbance change (Dekker et al., 1984a,b). The spectrum and the initial amplitude of absorbance changes caused by the flash were essentially the same in Cl^--depleted and in Cl^--reconstituted PS II (Figure 3). In agreement with XANES edge shifts and H^+

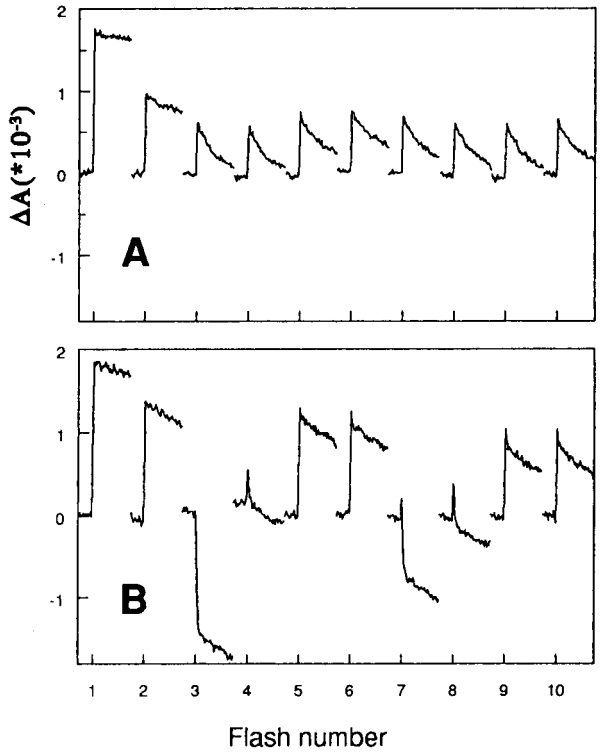


FIGURE 2: PS II donor side absorption changes recorded at 295 nm. (A) Cl^--depleted PS II membranes. (B) Cl^--reconstituted PS II membranes. In both cases samples were incubated 15 min at pH 7.5 in the presence of 50 mM Na_2SO_4. The measurements were performed immediately after incubation and addition of artificial electron acceptors (ferricyanide and DCBQ). In B, 50 mM NaCl was added to restore oxygen-evolving activity. Each trace represents 300 ms.

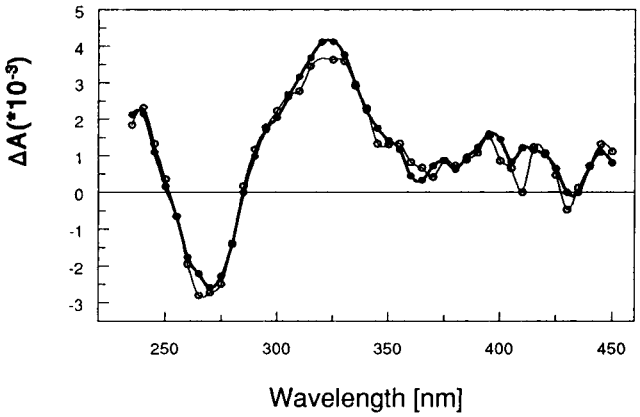


FIGURE 3: Absorption difference spectra induced by the first saturating flash in Cl^--depleted (heavy curve, solid circles) and Cl^--reconstituted (thin curve, open circles) PS II membrane fragments. The points for the plots are taken from the amplitude of the initial absorption changes. Measurements were performed at pH 7.5.

release measurements, the $S_1 \rightarrow S_2$ transition probed by the light-induced absorbance change at 295 nm does not require Cl^-, and the optical spectra for the two samples indicate that the component oxidized is the same chemical species in both cases. On the other hand, the S_2-state formed in the absence of Cl^- differs from the normal S_2-state in that its decay to S_1 is about 20 times slower (data not shown), in agreement with other results [see Coleman (1990)]. Also, a very small absorbance decrease is detected in the first milliseconds after the first flash, which is unique to Cl^--depleted samples (Wincencjusz et al., 1995).

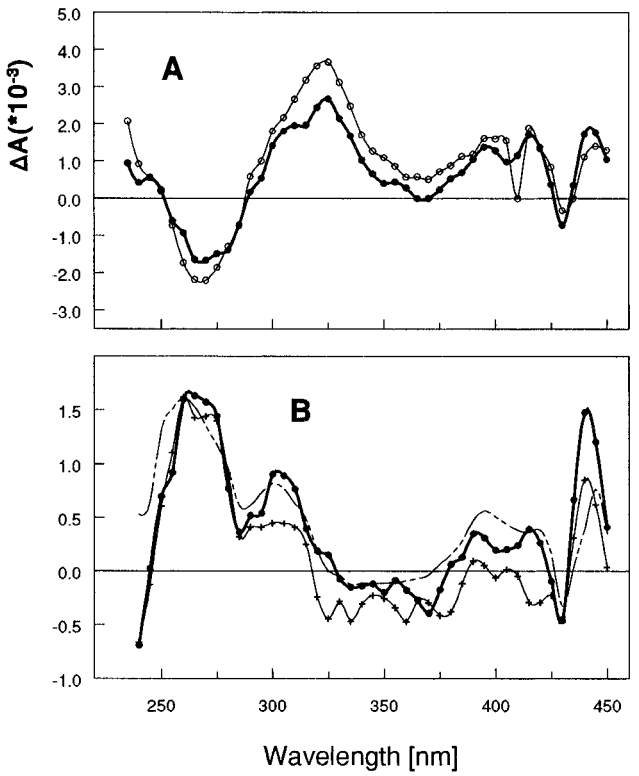


FIGURE 4: (A) Absorption difference spectra induced by the second flash in Cl^--depleted (heavy curve, solid circles) and Cl^--reconstituted (thin curve, open circles) PS II membranes. (B) Donor side contribution to the spectra induced by the second (heavy curve, solid circles) and subsequent flashes (thin curve, crosses) in Cl^--depleted PS II (these spectra were obtained after subtracting the $Q_A \rightarrow Q_A^-$ spectrum). Measurements were performed at pH 7.5. The dashed line represents the oxidized-minus-reduced spectrum of Tyr_Z in Mn-depleted PS II.

Cl^--Requirement of the $S_2 \rightarrow S_3$ Transition. Figure 2 shows that absorbance changes in Cl^--depleted PS II are clearly modified on the second and following flashes. Figure 4A shows the spectrum of the absorbance change induced by the second flash in Cl^--depleted (solid circles) and Cl^--reconstituted PS II (open circles) samples. In the presence of Cl^- the difference spectrum on the second flash is rather similar to that on the first in the absence or presence of Cl^-. In the absence of Cl^-, however, the second flash induced significantly different absorbance changes. These spectra indicate that in the absence of Cl^- the donor oxidized on the second flash is not the same species that is oxidized in the presence of Cl^-. By subtraction of the absorbance changes caused by Q_A reduction (taken from Dekker et al., 1984a) the difference spectra of the donor oxidized on the second and on the third flashes in the absence of Cl^- were estimated (Figure 4B). These spectra were clearly different from those reported for the normal $S_2 \rightarrow S_3$ transition by Lavergne (1991) and by van Leeuwen et al. (1993) and instead resemble the spectrum reported for tyrosine oxidation (positive bands at 260 and 300 nm and a small or negative amplitude at wavelengths between 320 and 370 nm; Dekker et al., 1984a). The total amount of $Y_Z^+Q_A^-$ formed after the second flash in Cl^--depleted samples appeared to be about 15% less than the amount of $S_2Q_A^-$ formed on the first flash. This difference is most likely due to the fraction of centers which contribute to the absorbance changes on the first flash only because electron transfer beyond Q_A^- is blocked (Lavergne, 1993). The fact that the decay of the absorbance increase at 295 nm after the second flash appears to be slower than that after subsequent flashes may be due to oxidation of any reduced Y_D as soon as a long-lived Y_Z^+ is formed (van Leeuwen et al., 1993).

In summary, these data indicate that after Cl^--depletion Y_Z^+ cannot oxidize S_2 to S_3 and instead decays by charge recombination with Q_A^-. The lifetime of the state $S_2Y_Z^+$ is unusually long (Ono et al., 1986a), with a half-time of 0.5 s (data not shown), perhaps in agreement with the slow deactivation of S_2 after one flash. The observation that Y_Z^+ cannot oxidize S_2 to S_3 in the absence of Cl^- is in agreement with a number of observations from other spectroscopic methods summarized in the introduction [see, however, Coleman (1990) and Boussac et al. (1994)]. A consequence of these findings is that the Cl^- dependence of the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition has not been established since S_3 cannot be formed in the absence of the halide.

Cl^--Requirement of the $S_3 \rightarrow S_0$ Transition. In order to determine if S_3 can advance to S_0 in the absence of Cl^-, certain conditions must be met. The anion must be removed after flash illumination to produce the S_3-state and depletion must occur on a time scale that is shorter than the intrinsic lifetime of S_3. Intact PS II membranes require only μM concentrations of Cl^- for full activity at pH 6.0. On the other hand, they lose Cl^- rapidly when the pH is raised to 8.5 and 50 mM Na_2SO_4 is added to the incubation medium. A dark-adapted, intact PS II sample, suspended in SM buffer (20 mM MES, pH 6.0, 0.4 M sucrose, less than 40 μM Cl^-) was advanced to the S_3-state by two flashes. Immediately afterward, a Tricine−sulfate solution with or without Cl^- was injected to obtain a final pH of 8.5, 50 mM sulfate, plus or minus 0.2 M NaCl. Injection and mixing took 5−10 s, and 15 s after the preilluminating flashes the measuring flash series was initiated. Results are shown in Figure 5. Figure 5A shows that under the conditions of pH (8.5) and ionic strength (200 mM each of NaCl and Na_2SO_4) used in these experiments, substantial period four oscillations are observed, indicating that these conditions do not block S-state cycling. Figure 5B,C shows the results obtained when an intact PS II membrane preparation was exposed to two preflashes before additions. By comparison to a control where Cl^- was included in the injection buffer (Figure 5C), it is clear that in the absence of Cl^- (Figure 5B) the period four oscillation and the transients due to $S_3 \rightarrow S_0$ transition were largely suppressed. The slowly decaying absorbance changes suggest that $Y_Z^+Q_A^-$ is still formed and decays by back reaction in most centers. The phase of the absorbance oscillation after addition of Cl^- (Figure 5C) indicates that about 50% of the S_3-state remained after the 15 s period of dark adaptation and reagent additions. If this is also true for the experiment shown in 5B, these measurements show that the $S_3 \rightarrow S_0$ transition requires the presence of Cl^-.

The data of Figure 5 do not exclude the possibility that the S_3-state becomes labile after removal of the halide and decays completely before the measuring flashes are fired. The resulting S_2-state would be unable to advance further (Figure 2). This possibility was examined, and the results are shown in Figure 6. The control experiment shown here (Figure 6B) is the same as that shown in Figure 5C with the exception that the dark incubation time between the preilluminating and measuring flashes was increased to 30 s. In the measurement shown in Figure 6A, 0.2 M Cl^- was not included in the Tricine−sulfate injection but was instead

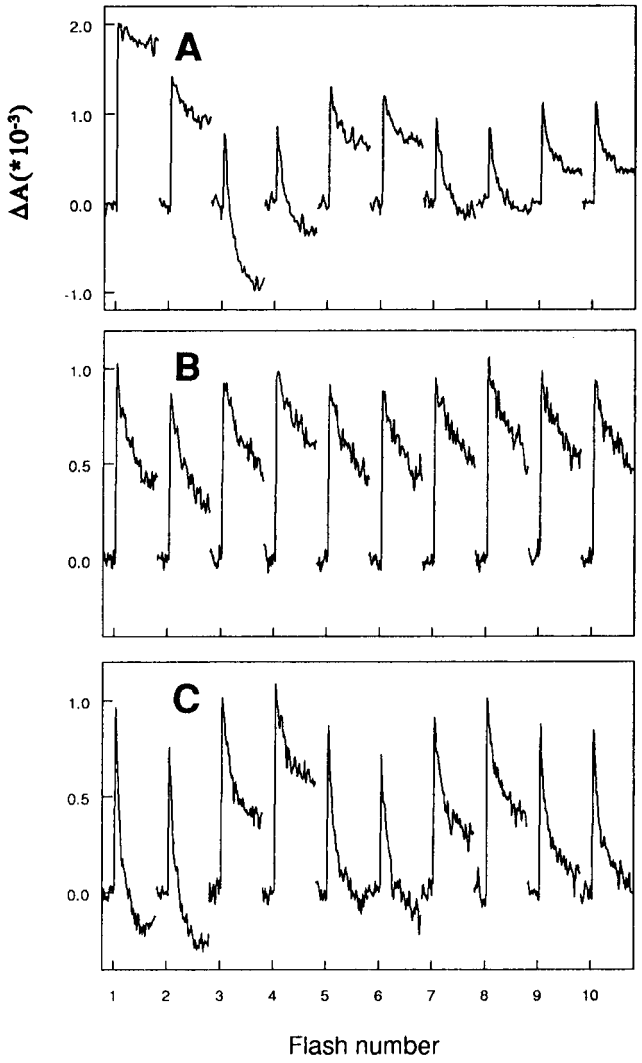


FIGURE 5: Effect of Cl^- depletion/readdition on donor side absorption changes recorded at 295 nm. Chloride depletion and readditions were carried out as described in Materials and Methods. (A) period 4 oscillation of Cl^--reconstituted PS II membranes at pH 8.5. The sample was incubated 10 min at elevated pH before the start of the measurement. (B) 295 nm absorption changes from a sample Cl^--depleted after two preflashes. The measurement started 15 s after the last preilluminating flash. (C) As in B but with 0.2 M NaCl included in the addition to the measuring cuvette.

added separately, 20 s after the first addition of Tricine/Na_2SO_4. The extent of the $S_3 \rightarrow S_0$ transition after the first flash is similar to that in Figure 6B. Also, the phase of the oscillation of the final absorption level after each trace is similar; the slightly lower level reached after flash numbers 2 and 6 reflects the inhibition of S_2 deactivation in the absence of Cl^-. The results presented in Figure 6 demonstrate that under the conditions of pH and ionic strength used here for rapid Cl^- depletion, the lifetime of the S_3-state, in contrast to that of S_2, is neither significantly lengthened nor shortened by the removal of Cl^-. These control experiments therefore support the conclusion derived from the results shown in Figure 5, namely, that Cl^- is an obligatory cofactor for the $S_3 \rightarrow S_0$ transition.

Cl^--Requirement of the $S_0 \rightarrow S_1$ Transition. The protocol described in Figure 5, but with three instead of two preilluminating flashes, was used to determine the Cl^--requirement of the $S_0 \rightarrow S_1$ transition. The results are shown in Figure 7. The first two flashes produced similar initial absorbance changes in both Cl^--depleted and Cl^--sufficient

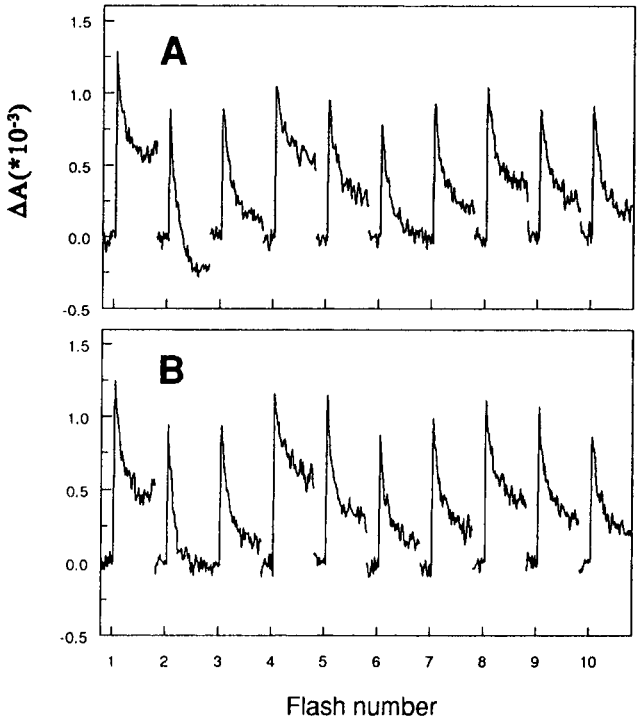


FIGURE 6: The comparative stability of the S_3-state in the absence (A) and presence (B) of Cl^-. The measurements were performed as in Figure 5 (two preflashes) except that the dark time was extended to 30 s. (A) 0.2 M NaCl was added 20 s after the second preilluminating flash. (B) 0.2 M NaCl was added simultaneously with Tricine and Na_2SO_4 immediately after the two preilluminating flashes.

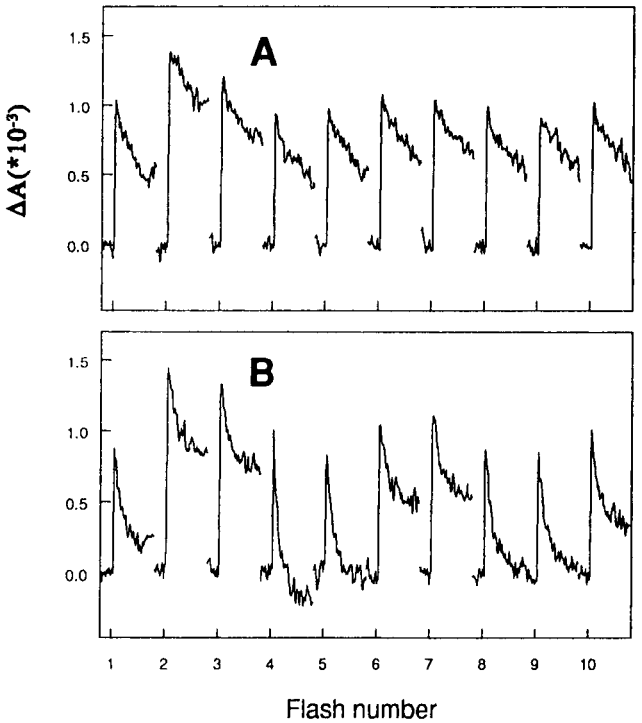
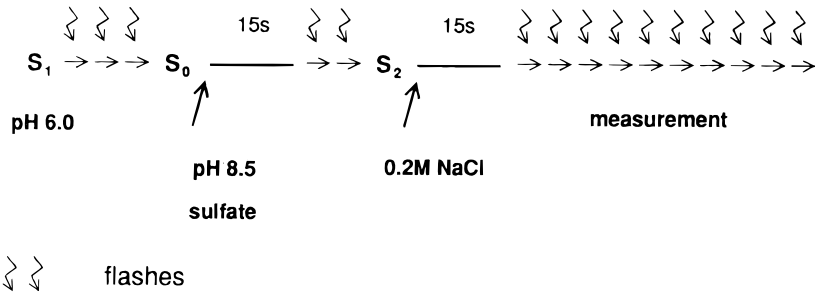


FIGURE 7: Cl^- requirement for the $S_0 \rightarrow S_1$ transition. Chloride depletion of the S_0-state was achieved by Tricine/SO_4^{2-} injection after three preilluminating flashes. The measurement started 15 s after the last preilluminating flash. (A) Cl^--depleted sample. (B) Cl^--reconstituted sample supplied with 0.2 M NaCl.

samples. The absorbance increase on the second flash is substantially larger than that on the first, as would be expected for the absorbance change due to $S_1 \rightarrow S_2$ relative to that of $S_0 \rightarrow S_1$ (Lavergne, 1991; van Leeuwen et al., 1993),

Scheme 1



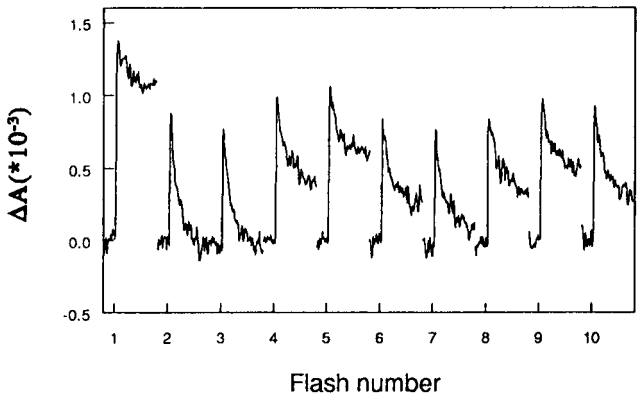


FIGURE 8: Cl^- requirement of the $S_0 \rightarrow S_1$ transition. The measurement was performed as illustrated in Scheme 1.

and also larger than these on later flashes, which are attributed to $Q_A^- Y_Z^+$ formation and recombination. The difference in decay kinetics for the absorbance transients shown in this experiment is attributed to the fact that centers missed on one or more preilluminating flashes undergo an $S_3 \rightarrow S_0$ transition in the presence of Cl^-. This is no longer possible after removal of the halide. The data of Figure 7 suggest that the S_0-state can be oxidized to S_1 in the absence of Cl^-, and that the resulting Cl^--depleted S_1-state can be advanced to S_2. The latter reaction is in accord with the data (Figure 2) showing that the halide is not required for the $S_1 \rightarrow S_2$ transition.

The lack of a Cl^- requirement for the $S_0 \rightarrow S_1 \rightarrow S_2$ transitions was verified by carrying out a measurement following the protocol shown in Scheme 1, starting with intact PS II membranes. If the $S_0 \rightarrow S_1$ transition does not require Cl^- and S_1 can advance to S_2 after Cl^--depletion, the sequence of manipulations outlined in Scheme 1 should result in synchronization of the majority of centers in the S_2-state. If this is so, then the $S_3 \rightarrow S_0$ transition should be detected on the second flash of the measuring series, whereas it should be more pronounced on the fourth flash if S_0 cannot advance in the absence of Cl^-. Results from such an experiment are shown in Figure 8. The oscillation is similar to that of a control sample beginning on flash number 6 (see Figure 5A) and confirms that most of the centers were indeed in the S_2-state at the beginning of the train of measuring flashes.

DISCUSSION

S-State Advancement in the Absence of Cl⁻. The UV absorbance difference spectra presented in Figures 3 and 4 present evidence for a $Mn^{III} \rightarrow Mn^{IV}$ oxidation state advancement on the $S_1 \rightarrow S_2$ transition on the first flash in dark-adapted, Cl^--depleted PS II membranes, whereas succeeding flashes induce $Q_A^- Y_Z^+$ formation and recombination. These findings are consistent with data from other laboratories showing that Cl^- depletion blocks advancement from S_2 to S_3. The observation that P_{680}^+ reduction, measured by the μs rise of Chl fluorescence, remains fast only one flash beyond the S_2-state was interpreted to indicate the formation of a long-lived Y_Z^+ on that flash (Itoh et al., 1984). An extensive investigation of tyrosine radical lifetimes in Cl^--depleted PS II (Deak et al., 1994) indicated the formation of a substantial amount of long-lived Y_Z^+ from the second flash on. Likewise, recent results from XANES studies (Ono et al., 1995) are in good agreement with the data we present here; in Cl^--depleted PS II membranes, the Mn K-edge energy is upshifted on the $S_1 \rightarrow S_2$ transition but does not undergo significant changes on subsequent flashes. This implies that a Mn^{III} to Mn^{IV} oxidation occurs in Cl^--depleted material on the first flash, but further metal-centered oxidation state advancements are blocked. The inhibition of S-state advancements beyond S_2 in the absence of Cl^- was also detected in EPR experiments on Cl^--depleted S_2 reported by Ono et al. (1986b); the amplitude of the S_2 multiline signal formed by dark Cl^- addition was the same after one or more flashes.

In contrast to the results just cited, other authors have reported advancement to the S_3-state in Cl^--depleted PS II (Boussac et al., 1992; Baumgarten et al., 1990). It is possible that this abnormal S_3-state is equivalent to the state $S_2 Tyr^+$. On the basis of their EPR data, Boussac et al. (1994) attributed the first electron transfer event in Cl^--depleted PS II membranes to the oxidation of an amino acid residue the authors propose to be histidine [see also Boussac et al. (1990)] and the second to oxidation of manganese. The authors do not exclude, however, an alternative explanation of their data which would involve EPR-silent manganese oxidation on the $S_1 \rightarrow S_2$ transition that reverts to a paramagnetic state upon S_3 formation. Partial support for this view came from UV absorption difference measurements on Cl^--depleted PS II by Haumann et al. (1995). In accord with the interpretation we present for our data, Haumann et al. conclude that only tyrosine oxidation occurs on the second flash in Cl^--depleted PS II. In contrast, however, their $S_1 \rightarrow S_2$ spectrum in Cl^--depleted material resembles the $S_2 \rightarrow S_3$ spectrum in Cl^--reconstituted PS II and differs from the $S_1 \rightarrow S_2$ spectrum in Cl^--reconstituted PS II [a much smaller signal amplitude around 350 nm, see Boussac et al. (1990)]. Haumann et al. interpret this to indicate histidine rather than manganese oxidation. However, their spectra for $S_2 \rightarrow S_3$ in Cl^--reconstituted preparations and $S_1 \rightarrow S_2$ in Cl^--depleted preparations differ substantially from $S_2 \rightarrow S_3$ spectra reported by Lavergne (1991) and by van Leeuwen et al. (1993) in the following way: the 350 nm shoulder is missing and a new band appears around 400 nm . Our data may

also suggest differences with published $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ spectra, but their significance and origin are not obvious; the large acceptor side contribution and different pH make any detailed analysis rather uncertain. Figure 3 does show, however, that in this case the spectrum on the first flash does not depend on the presence of Cl^-.

The rationalization presented by Haumann et al. (1995) for histidine rather than manganese oxidation on the first flash is that Cl^- removal would increase the midpoint potential of the S_2/S_1 redox couple above that needed for histidine oxidation. This is difficult to reconcile with both the slower S_2 deactivation (Ono et al., 1986b) and estimations of redox potentials for S_2/S_1 measured by thermoluminescence (Vass et al., 1987) in Cl^--depleted PS II membranes. In the latter experiments, the upshift of the recombination temperature of the $S_2Q_A^-$ and $S_2Q_B^-$ pairs has been attributed to a reversible decrease (60−80 mV) of the S_2/S_1 midpoint potential after Cl^--depletion which is consistent with the about 20-fold slower decay of S_2 by charge recombination (Coleman, 1990).

Our data reveal no difference between the $S_1 \rightarrow S_2$ spectra obtained with or without Cl^-. We conclude that in the absence of Cl^-, Y_Z^+ can oxidize the manganese cluster from S_0 to S_1 and from $S_1 \rightarrow S_2$ (Figures 7 and 8) but that subsequent S-state transitions are blocked. This model can be rationalized by assuming that a single Mn atom is directly accessible to oxidation by Y_Z^+. Distinguishing this "proximal" Mn from "distal" Mn by the subscripts "p" and "d", respectively, would imply that Mn_p can store only 2 oxidizing equiv (the Mn^V oxidation state is not considered to be biologically accessible) and probably consists of one Mn that changes oxidation state from Mn^{II} to Mn^{IV} on $S_0 \rightarrow S_1 \rightarrow S_2$. The Mn oxidation state assignment for S_1 derived from XANES data (Riggs et al., 1992; Yachandra et al., 1993) can be expressed as 2 $Mn_{p,d}{}^{III}$/2 Mn^{IV} so that in the presence of Cl^-, the S_2-state following a flash would be $Mn_p{}^{IV}/Mn_d{}^{III}$, and the following dark reaction, $Mn_p{}^{IV}/Mn_d{}^{III} \rightarrow Mn_p{}^{III}/Mn_d{}^{IV}$, would return Mn_p to the lower oxidation state which is oxidizable by Y_Z^+ on the $S_2 \rightarrow S_3$ transition. If such an intermetal redox reaction were dependent on the presence of Cl^- acting either as a ligand or as a factor to neutralize charge, then the Cl^--depleted S_2-state would contain a $Mn_p{}^{IV}$ that cannot be further oxidized by Y_Z^+, inducing a block at the $S_2 \rightarrow S_3$ transition. It is not obvious that such an hypothesis would be compatible with the absence of a Cl^- effect on the $S_1 \rightarrow S_2$ difference spectrum. In the absence of Cl^- its binding site is presumably occupied by the small, hard ligand OH^- (Homann, 1988). This could inhibit electron transfer from Mn_d to Mn_p either mechanistically, e.g., if a Cl^- bridge between Mn_p and Mn_d were disrupted, as proposed by Sandusky and Yocum (1984), or thermodynamically, if Cl^- were a terminal ligand (Yachandra et al., 1993) to Mn_p only and replacement by OH^- lowers the $Mn_p{}^{IV}/Mn_p{}^{III}$ potential substantially below that of Mn_d, in accord with the unusual stability of the S_2-state in the absence of Cl^-. However, the estimated 80 mV decrease in S_2/S_1 midpoint potential alone seems insufficient to explain the block of the S-state advancements beyond the S_2-state because the lifetime of $Y_Z^+S_2$ in the absence of Cl^- is much longer than 20 times the time it takes to proceed to Y_ZS_3 in the presence of Cl^-. To assume in addition that Cl^--depletion raises the potential of S_3/S_2 above that of Y_Z^+/Y_Z seems unreasonable in view of the unchanged S_3 lifetime and the large, slowly decaying absorbance changes still observed (Figure 5B). If Cl^--depletion had changed Y_ZS_3 into $Y_Z^+S_2$ only $P_{680}^+Q_A^-$ recombination would be expected in the majority of the centers. Therefore, to explain the observed effects of Cl^--depletion it is probably necessary to assume that both mechanistic and thermodynamic factors play a role. An intervalence redox rearrangement has been proposed by Boussac et al. (1996) to explain the transition between the $g = 2$ multiline signal and the $g = 4.1$ EPR signal associated with the S_2-state. It is conceivable that the effects of Cl^--depletion on formation of these signals (Britt, 1996) may be relevant to the redox rearrangements between Mn atoms in the OEC proposed here and in Boussac et al. (1996), although it is unclear at the present time how our proposal for Mn oxidation state changes would relate to formation of the $g = 4.1$ signals elicited by various inhibitory treatments of the OEC.

Cl^- is essential for S-State Advancements above S_2. The ability to quickly deplete PS II membranes of Cl^- and to restore high concentrations of the halide have allowed us to determine its requirement for each of the S-state transitions. As the data in Results show, both $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions require Cl^-. The preillumination/Cl^--depletion/reconstitution methods employed here demonstrate that although the S_2-state formed in the absence of Cl^- may possess abnormal EPR properties (Ono et al., 1986b), it can revert quickly (<15 s) in the dark to a form that exhibits normal advancement properties. There are no spectroscopic data that describe the properties of S_3 in the Cl^--depleted state; our results suggest that the lifetime of this state is not dramatically altered by Cl^- depletion.

The essential participation of Cl^- in the redox reactions that immediately precede O_2 production by PS II indicate that this cofactor may be critically important in controlling Mn redox reactions that catalyze water oxidation. If Cl^- were to function exclusively as a counter anion to suppress positive charge accumulation in the Mn cluster, it is curious that such neutralization seems unnecessary for oxidation of the lowest two S-states. This observation suggests that either Mn-centered oxidation events or sequential oxidation of bound H_2O molecules, as proposed in some models (Hoganson et al., 1995; Babcock, 1995), can occur independently of the presence of Cl^-. In view of the results we present here, these models may require revision.

Although our data add to an extensive literature providing evidence that Cl^- is unnecessary for the $S_1 \rightarrow S_2$ transition, other results indicate that the anion is present in the OEC throughout the S-state cycle. The data of Lindberg and Andréasson (1995) show quite convincingly that in the S_1-state, Cl^- is in fact a tenaciously bound cofactor whose retention is strongly facilitated by the presence of the extrinsic 23 and 17 kDa polypeptides. High-affinity binding of Cl^- throughout the S-state cycle is evidenced by the ability of intact PS II membranes under continuous illumination to maintain robust rates of O_2 evolution activity in assay media containing little or no added Cl^-. It is possible that the halide remains bound to the same site in all S-states, but is required only in the higher S-states as an essential cofactor for the oxygen-evolving reaction.

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