The Function of the Chloride Ion in Photosynthetic Oxygen Evolution†

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Received May 22, 2002; Revised Manuscript Received December 4, 2002

ABSTRACT: The involvement of Cl^- and several other monovalent anions in photosynthetic oxygen evolution was studied using photosystem II membranes depleted of Cl^- by dialysis. The results of these studies differ significantly from results obtained using other depletion methods. Binding studies with glycerol as a cryoprotectant confirm our previous observations with sucrose of two interconvertible binding states of photosystem II with similar activities and with slow or fast exchange, respectively, of the bound ion. With glycerol, Cl^- depletion decreased the oxygen evolution rate to 55% of that with Cl^- present without decreasing the quantum efficiency of the reaction, supporting our previous conclusion that oxygen evolution can proceed at high rates in the absence of Cl^-. Further, after Cl^- depletion the S_2 state multiline signal displayed the same periodic appearance with the same signal yield after consecutive laser flashes as with Cl^- present. Br^-, I^-, and NO_3^-, although with different capacities to reactivate oxygen evolution, also showed two binding modes. I^- inhibited when bound in the low-affinity, fast-exchange mode but activated in the high-affinity mode. A comparison of the EPR properties of the S_2 state with these anions suggests that the nature of the ion or the binding mode only has a minor influence on the environment of the manganese. In contrast, F^- completely inhibited oxygen evolution by preventing the S_2 to S_3 transition and shifted the equilibrium between the $g = 4.1$ and multiline S_2 forms toward the former, which suggests a considerable perturbation of the manganese cluster. To explain these and earlier observations, we propose that the role of chloride in the water-splitting mechanism is to participate together with charged amino acid side chains in a proton-relay network, which facilitates proton transfer from the manganese cluster to the medium. The structural requirements likely to be involved may explain the sensitivity of oxygen evolution to Cl^- depletion or other perturbations.

Photosynthetic water oxidation catalyzed by photosystem II (PS II)[1] depends on the coordinated abstraction of electrons and protons from substrate water by a tetranuclear cluster of manganese ions in the oxygen-evolving complex (OEC). In addition to manganese the inorganic cofactors Ca^{2+} and Cl^- have been observed to be involved in water oxidation by the OEC. Chloride has been variously suggested to function as a ligand to the manganese cluster, as a bridge between calcium and the Mn cluster, or to compensate for charges accumulated by the manganese cluster during turnover (*1*, *2*). There is evidence that in the absence of Cl^- stabilization as well as modification of the S_2 state occurs (*3*). It has also been suggested that Cl^- stabilizes the structure of OEC after depletion of the 17 and 23 kDa proteins (*4*, *5*). An apparent correlation between reactivation of oxygen evolution in Cl^--depleted centers by monovalent anions and their ionic radius and electric field strength has been noticed (*6*). This and the limited specificity of the anion site put restrictions on the involvement of Cl^- in the reaction mechanism.

The requirement for Cl^- in photosynthesis was studied already by Warburg (*7*) and has been the subject for continued interest ever since. Some early observations where extensive Cl^--free washing was used to remove Cl^- from thylakoids pointed to a significant residual oxygen-evolving activity in the depleted material (*8*, *9*). However, this time-consuming depletion method was soon replaced by quicker and more convenient procedures involving uncouplers, high pH, and sulfate. An important role for Cl^- in PS II catalyzed water oxidation has been taken for granted as such treatments used to remove Cl^- in most cases lead to more or less severe inhibition of the oxygen-evolving activity, which may be recovered by the addition of Cl^- (the "chloride effect"). One problem with most depletion methods used is that they generally result in an increase of the Cl^- requirement in comparison with untreated PS II membranes by sometimes as much as several orders of magnitude. The reason can often be traced to a more or less extensive and irreversible loss of extrinsic proteins associated with the PS II lumenal side (*9*), which can be compensated for by high concentrations of Cl^- (*5*), or to more subtle irreversible changes in the properties of PS II (*5*). The importance of irreversible changes for the chloride effect was pointed out already by Wydrzynski et al. (*10*). Thus, depending on the method used for Cl^- removal, studies of the anion dependence of oxygen evolution in PS II have often been carried out with preparations more or less severely altered. In some cases (see, for example, ref

† This work was supported by a grant from the Swedish Natural Science Council.

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[1] Abbreviations: Chl, chlorophyll; PPBQ, phenyl-*p*-benzoquinone; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; PS II, photosystem II.

10.1021/bi026175y CCC: $25.00
Published on Web 01/28/2003

11), the anion dependence of PS II activity was determined after the extrinsic proteins had been purposely removed to eliminate possible heterogeneities in composition and reactivity. When the mechanism of Cl^- action is discussed, it should be remembered that results obtained might be relevant only to the specific state of the photosynthetic material used, a state, which is strongly dependent on previous treatment of the preparation.

Since, over the years, most studies of the role of Cl^- in PS II have been carried out with photosynthetic material with an increased Cl^- dependence, questions may be asked about the actual involvement of Cl^- in PS II under more natural conditions. More recently, our laboratory used extensive dialysis as an alternative method for complete removal of Cl^- from isolated PS II membranes to preserve as much as possible the integrity of the native membranes (*12*, *13*). Our analysis from the Cl^- binding experiments showed that a single Cl^- ion is normally trapped by a tight-binding (closed) state of PS II from which the ion is released only very slowly concomitantly with conversion to a weak-binding (open) state of PS II. On binding of Cl^- to the open state a slow conversion to the closed state occurs. The Cl^--deficient, open state in sucrose-containing buffer at pH 6.3 retained about 30% of the activity observed with saturating concentrations of Cl^-. An analysis of the light dependence of the oxygen evolution in membranes Cl^--depleted by dialysis showed that depletion lowered the rate at saturating light without affecting the quantum efficiency of the reaction. This strongly indicates that the residual activity arises from the depleted centers, a result in obvious disagreement with the widely held view that Cl^- is an indispensable component in the water oxidation reaction, and forces a reassessment of the role of Cl^- in the mechanism of photosynthetic oxygen evolution.

Taking advantage of the preserving nature of the dialysis method of Cl^- removal in comparison with other methods, we have compared binding properties with glycerol as a cryoprotectant to earlier published binding data using sucrose and examined the ability of different monovalent anions, such as Br^-, I^-, NO_3^-, and F^-, to reactivate Cl^--depleted PS II preparations. Comparisons have been made with Cl^- with regard to affinity, kinetics of binding and dissociation, the conversion between strong and weak binding, and effects on EPR properties, all in search for behavioral patterns, which may help to elucidate the role of Cl^- in photosynthetic oxygen evolution closer to native conditions.

With the significant residual activity of PS II membranes depleted by dialysis one expects that cycling of the S-state intermediates should be detectable and accessible for analysis. Here we have carried out experiments using the multiline EPR signal of the S_2 state to monitor S-state turnover in Cl^--depleted and anion-reconstituted PS II membranes, illuminated by short laser pulses. Finally, given the results of our studies and data by others, we present a hypothesis of a possible role for Cl^- in the water-splitting reaction.

MATERIALS AND METHODS

Photosystem II. PS II membranes were prepared from hydroponically grown spinach after dark adaptation for 0.5 h following harvest, using the procedure described in ref *14*, and frozen in liquid nitrogen as droplets for storage at 77 K. The oxygen-evolving activity of the PS II membranes was typically in the range of 750−950 μmol of O_2 (mg of Chl h)$^{-1}$ at saturating light. Chloride removal was carried out by repetitive washing followed by dialysis (Spectra/Por, MW cutoff 50000 Da) (*15*) in the dark at 0 °C for 10 h against Cl^--free 20 mM MES−NaOH buffer, pH 6.3, with glycerol, 146.7 g L^{-1} (1.4 M), as a cryoprotectant. This buffer with appropriate concentrations of Na salts of various monovalent anions was used throughout this study. The Cl^- content in all Cl^--free buffers used was measured using the procedure given in ref *15* to reveal any traces of contaminating Cl^-. The level of contamination was routinely found to be less than the detection limit, 1 μM.

Oxygen Evolution Activity Measurements. Steady-state oxygen evolution activities were measured at 20 °C with a Clark-type oxygen electrode coupled to an Oxytherm monitor (Hansatech). The electrode was calibrated in pure water with corrections for partial pressure and temperature. To ensure that correct readings were obtained in buffer containing glycerol, the oxygen signals were compared after addition of a known amount of oxygen to deoxygenated water or buffer. No significant differences in the readings were observed. Near-saturating, white light from a 250 W tungsten−iodide lamp was passed through a 0.1 m water filter and a Balzer B_1K_1 heat reflectance filter. With saturating light we observed that the activity of the Cl^--depleted PS II membranes was gradually lost during a run, resulting in curved traces. This is probably a result of damage to the donor side caused by slower electron transfer from water in the absence of Cl^-. To prolong the linearity of the reaction curves and facilitate the rate measurements, a somewhat lower light intensity (80% saturation) was used. All measurements were done using 20 mM MES−NaOH buffer, pH 6.3, and 1.4 M glycerol and with 1.25 mM PPBQ as an electron acceptor and with appropriate concentrations of Na salt(s) added as indicated in the figure legends.

Determination of Anion Binding Parameters. The dissociation constants for anion binding to PS II were determined as described in ref *13*. For the determination of the dissociation constant for the low-affinity anion binding (O-state) of PS II a small aliquot of Cl^--depleted membranes was added to the assay medium supplemented with the appropriate concentration of anion (Na salt). The oxygen evolution rate was measured within 10 s after the addition of the PS II sample to the assay vessel and followed for at least 2 min. The dissociation constant was calculated from the dependence of the rate on the concentration of anion. The dissociation constant for the high-affinity C-state was determined from oxygen evolution rates obtained with PS II membranes (1.5 mg of Chl mL^{-1}), preincubated on ice (2 h) with the anion at the appropriate concentration, and then mixed with the anion-free buffer in the oxygraph chamber (dilution factor 70) and measured within 10 s. The oxygraph traces were followed for at least 2 min.

The rate constant of anion binding in the closed state was determined as follows: Chloride-depleted PS II membranes (1.5 mg of chlorophyll mL^{-1}) were incubated at 0 °C in the dark in an anion-containing (200 μM) medium (Na salt). Samples, taken at various times after the start of the incubation, were then diluted 70-fold in the anion-free assay medium, and the oxygen-evolving activity was recorded within 10 s. The observed rate constant was determined from the incubation time dependent increase in activity, assuming

a monoexponential reaction.

To determine the rate constant for the dissociation of anions from the closed state, PS II membranes were first incubated for 2 h at 0 °C in darkness at a chlorophyll concentration of 1.5 mg of Chl mL^{-1} in buffer with the anion containing (200 μM) to be investigated. Small aliquots were then withdrawn from the incubation mixture and diluted 70-fold into the anion-free medium in the measuring vessel. The oxygen evolution activity was recorded after different times of incubation in the measuring vessel and followed for at least 2 min. For determination of the rate constant for the dissociation of fluoride, a sample incubated with 25 mM fluoride (2 h on ice in darkness) was quickly washed several times with Cl^--free buffer. From the washed, F^--containing membranes, samples were withdrawn after various times for measurement of the activity after 70-fold dilution in the anion-free assay medium. The rate constant was determined from the time-dependent increase in activity as F^- dissociates from its binding site.

EPR Measurements. X-band EPR spectra from the S_2 state of the OEC were recorded with a microwave frequency of 9.34 GHz at a microwave power of 20 mW and a temperature of 11 K. The S_2 state was generated by continuous illumination for 4 min at 200 K (*16*) of dark-adapted, Cl^--depleted PS II membranes after incubation with 10−25 mM Na salt of selected anions. Flash illumination at 532 nm was accomplished by giving 150 mJ light flashes from a frequency-doubled YAG laser (Spectra Physics, GCR 190-10) to dark-adapted, Cl^--depleted, and anion-reconstituted PS II membranes at 3 mg of Chl/mL in EPR tubes. The samples were first given a synchronizing laser flash (*17*) and allowed to relax in the dark for an additional 5 min at 0 °C before being subjected to one or more saturating flashes at 1 Hz. After flash illumination the samples were quickly frozen in dry ice−ethanol followed by cooling to 77 K before measurement.

SDS−PAGE. PS II samples were reduced with 0.1 M dithiothreitol and solubilized in 5% SDS and 62.5 mM Tris-HCl, pH = 6,8. SDS−PAGE was performed according to ref *18* on 10−20% gradient polyacrylamide gels (Bio-Rad, Criterion). Protein bands were stained with 0.5% Coomassie Brilliant Blue R-250.

RESULTS

Polypeptide Composition of Cl-Depleted PS II Membranes. To compare the dialysis procedure used here for Cl^- depletion with the commonly used high pH−sulfate method in its ability to influence the integrity of PS II, the polypeptide composition of PS II membranes was investigated by SDS−PAGE. After dialysis of PS II membranes against a Cl^--free medium containing glycerol, no change in polypeptide composition was detected compared to the control (Figure 1, lane B), and restoration of the oxygen evolution activity could be achieved with Cl^- concentrations in the order of 20 μM (see below). Partial loss of extrinsic proteins in conjunction with a high demand for Cl^- (several millimolar) to restore oxygen evolution was observed after high pH−sulfate treatment (Figure 1, lane D). For comparison, the polypeptide pattern of PS II membranes depleted of the 17 and 23 kDa proteins by washing with 1 M NaCl is also shown (lane C). The protocols for removing extrinsic proteins by salt washing in lane C or high pH treatment in lane D were as described earlier (*19*) with the modification that glycerol was exchanged for sucrose. The incomplete removal of the 17 and 23 kDa extrinsic proteins observed in glycerol after treatment at high pH and sulfate (cf. ref *20*) might indicate a stabilizing effect of glycerol compared to sucrose. Further evidence of such stabilization was seen as a reduced loss of activity, resulting from repeated freezing and thawing of PS II membranes, which became negligible from about 10% loss on every freeze−thaw cycle. In addition, the individual traces in the oxygen evolution measurements became almost linear for 2 min in glycerol compared to a 30−50% gradual decrease in activity over the same time period in a medium with sucrose.

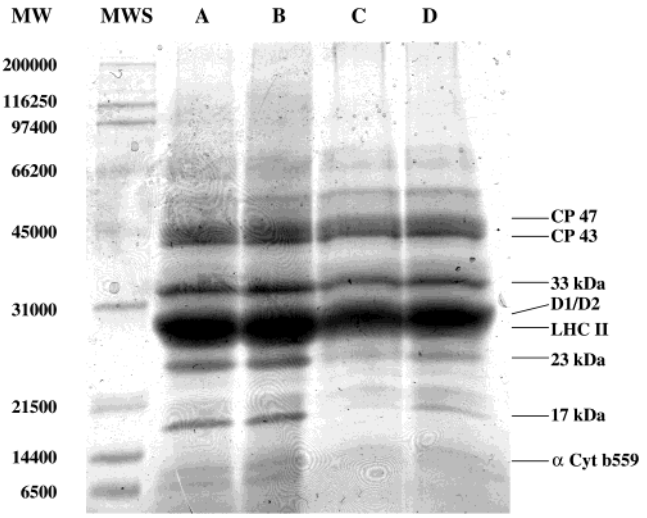

FIGURE 1: Influence of the Cl^- depletion method on the polypeptide composition of PS II membranes. SDS−PAGE was performed on a 10−20% gradient gel with PS II samples containing 11 μg of chlorophyll. Lanes: A, untreated PS II membranes; B, PS II membranes depleted of Cl^- by dialysis for 10 h against Cl^--free 20 mM MES−NaOH, pH 6.3, and 1.4 M glycerol; C, PS II membranes treated with 1 M NaCl in 20 mM MES−NaOH, pH 6.3, and 1.4 M glycerol; D, PS II membranes depleted of Cl^- by washing with 50 mM HEPES−NaOH, pH 7.5, 50 mM Na_2SO_4, and 1.4 M glycerol. MWS shows a molecular weight standard with the appropriate molecular weights indicated. The positions of several PS II proteins on the gel are indicated at the right.

Reactivation of Oxygen Evolution by Monovalent Anions. Since glycerol was used as a cryoprotectant in the present study instead of sucrose, the activity characteristics of Cl^--depleted and reconstituted membranes in this medium were reinvestigated. Dialyzed, Cl^--free photosystem II membranes in a glycerol-containing buffer show a considerable basal oxygen evolution activity (Figures 2A and 3), confirming results obtained earlier with Cl^--depleted PS II membranes in sucrose media (*12, 13*) although the remaining activity in glycerol was even higher or about 55% of that of untreated membranes in saturating light compared to 30% in sucrose. This seems to be a property peculiar to the cryoprotectant since transfer of depleted PS II membranes from a glycerol-containing buffer to one containing sucrose led to a drop in the oxygen-evolving activity while an increase was observed on the reverse transfer.

Oxygen evolution was instantaneously reactivated by addition of 10 mM Cl^- to dialyzed, Cl^--free membranes, saturating low-affinity binding (O-state), with K_d = 0.8 mM (Figure 2 A). At concentrations of Cl^- higher than about 25

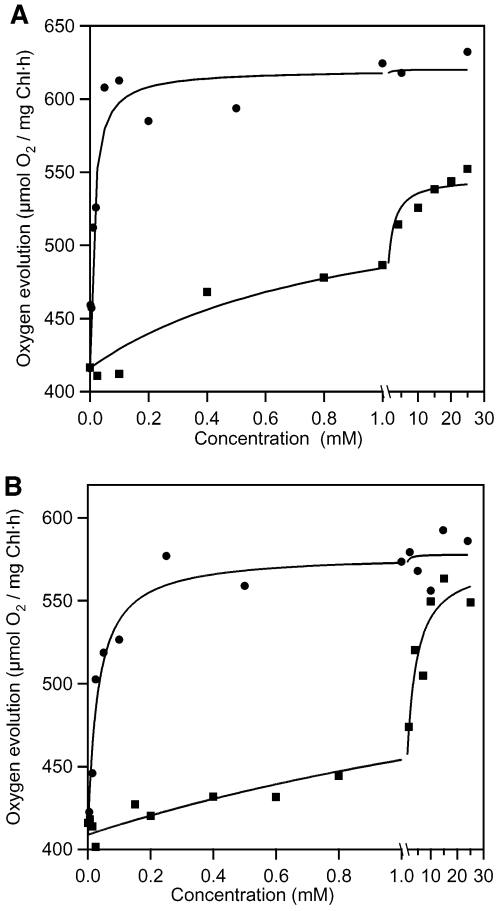

FIGURE 2: Anion-dependent recovery of oxygen evolution in Cl^--depleted PS II membranes. PS II membranes were dialyzed against Cl^--free buffer for 10 h in darkness at 0 °C. (A) (■) Activity measured 10 s after addition of the Cl^--depleted PS II membranes to the assay medium containing 20 mM MES−NaOH, pH 6.3, and 1.4 M glycerol and NaCl as indicated. (●) Activity of Cl^--depleted PS II membranes after incubation with NaCl for 2 h in darkness at 0 °C at the concentrations indicated in the figure but measured in Cl^--free buffer. (B) Bromide-dependent recovery of oxygen evolution in Cl^--depleted PS II membranes. (■) Activity measured 10 s after addition of the Cl^--depleted PS II membranes to the assay medium containing NaBr at the concentrations indicated. (●) Activity of Cl^--depleted PS II membranes after incubation with NaBr for 2 h in darkness at 0 °C at the concentrations indicated in the figure, measured in the absence of added anion.

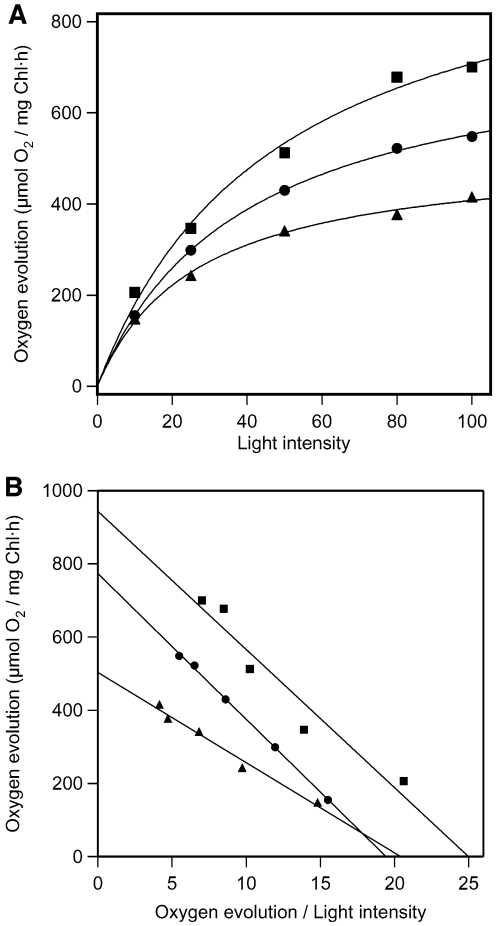

FIGURE 3: Effect of light intensity and Cl^- on the oxygen evolution rate in PS II membranes. (A) Light dependence of the oxygen-evolving activity in Cl^--sufficient and Cl^--depleted PS II membranes. (■) Control PS II membranes in 20 mM MES−NaOH, pH = 6.3, 1.4 M glycerol, and 15 mM NaCl. (▲) PS II membranes depleted of Cl^- by dialysis for 10 h against Cl^--free 20 mM MES−NaOH, pH = 6.3, and 1.4 M glycerol, measured in the same buffer. (●) Cl^--depleted PS II membranes in MES−NaOH buffer, pH = 6.3, and 1.4 M glycerol reactivated with 10 mM NaCl for 2 h. All measurements were done at a chlorophyll concentration of 20 μg mL^{-1} and 20 °C. (B) Data points from (A) replotted as a Scatchard plot.

mM inhibition was observed in the O-state. Restoration of oxygen evolution was also accomplished by incubation of Cl^--free membranes at low (micromolar) concentrations of Cl^- for 2−3 h, which resulted in a K_d value of 13 μM for binding in the high-affinity C-state.

The level of recovered oxygen evolution in the C-state was about 80% of the activity obtained with untreated membranes at saturating Cl^- concentrations. This indicates that an irreversible loss of oxygen evolution activity occurs in 20% of the centers as a result of the depletion procedure. A Scatchard plot of the activity of Cl^--depleted and reactivated PS II at different light intensities displays a common point of interception on the abscissa axis (Figure 3B), showing that the quantum efficiency of the reaction was unaffected by Cl^- depletion (*21*). This result is expected if the residual activity arises from Cl^--depleted centers and supports earlier results obtained using a sucrose-containing medium (*12*, *13*). Rapidly exchanging Cl^- serving several centers cannot account for the results since contaminating Cl^- would be trapped in the slowly exchanging C-state by virtue of its high affinity for Cl^-. In addition, the concentration of contaminating Cl^- is too low to cause an observable effect on the activity by binding in the low-affinity state.

The binding−reactivation properties of bromide were similar to those of Cl^- (Figure 2B), showing both low- and high-affinity binding with dissociation constants of 2.8 mM and 31 μM, respectively, consistent with the one-site, two-state binding model. However, the inhibition at high concentrations of Br^- in the O-state was not as evident as with Cl^- (cf. Figure 2). Other anions studied differed from Cl^- and Br^- with regard to their effects on anion-depleted membranes, showing less activation or even inhibition. A summary of the binding/dissociation studies may be found in Table 1. With I^- (Figure 4), a dissociation constant of 13 μM was found for the C-state, and restoration of oxygen evolution was less efficient than with Cl^- and Br^-. The binding of I^- in the O-state inhibited oxygen evolution in a biphasic fashion already at low concentrations with inhibitory

Table 1: Interaction of Anions with Cl^--Depleted PS II Membranes: Binding and Activation Properties of Selected Monovalent Anions

	O-state		C-state			
anion	K_d (M)	activity[a]	K_d (M)	activity[a]	k_{on}^{obs} (s^{-1})	k_{off}^{obs} (s^{-1})
Cl^-	8×10^{-4}	552	13×10^{-6}	618	1.4×10^{-3}	6.5×10^{-3}
Br^-	2.8×10^{-3}	586	31×10^{-6}	581	6.8×10^{-4}	17×10^{-3}
I^-	7×10^{-6},[b] 1.05×10^{-2} [b]	0	13×10^{-6}	537	8.9×10^{-3}	11×10^{-3}
NO_3^-	8×10^{-4}	521	2.8×10^{-3}	430	2.7×10^{-3}	9.4×10^{-3}
F^-	1.7×10^{-2} [b]	110	1.5×10^{-2} [b]	0	2.7×10^{-3}	2.6×10^{-4}

[a] The activity is expressed in units of μmol of O_2 (mg of Chl h)$^{-1}$ and was measured at a saturating concentration of the anion and with near-saturating light. The activity in the absence of additions was 416. [b] Inhibition constant.

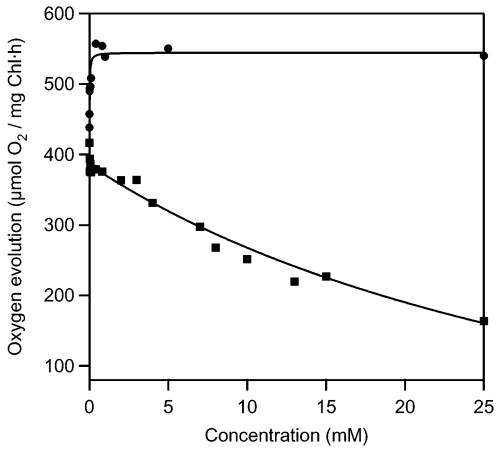

FIGURE 4: Iodide-dependent recovery of oxygen evolution in Cl^--depleted PS II membranes. (■) Activity measured 10 s after addition of the Cl^--depleted PS II membranes to the assay medium containing NaI at the concentrations indicated. (●) Activity of Cl^--depleted PS II membranes after incubation with NaI for 2 h in darkness at 0 °C at the concentrations indicated in the figure, measured in the absence of added anion.

constants of 7 μM and 10 mM, respectively. The minor (<10%) 7 μM phase could originate from a second binding site for I^-. Interestingly, no inhibition was observed after addition of 10 mM I^- to a sample reactivated with iodide tightly bound in the C-state.

Nitrate was found to be an overall poor substitute for Cl^-. First, the dissociation constant for NO_3^- was found to be 2 orders of magnitude higher than for Cl^- in the C-state after long time (2−3 h) incubation (Table 1). Second, the activity supported by NO_3^- was considerably lower than accomplished with Cl^-. The affinity for NO_3^- in the O-state was similar to that of Cl^-, but the magnitude of restored oxygen evolution activity 10 s after the addition of NO_3^- was less than half of that seen with Cl^-. When 10 mM Cl^- was added to a sample preincubated with NO_3^- for 2−3 h, no further rapid increase in oxygen evolution was observed, indicating competition between NO_3^- and Cl^- for a common site.

As described above, a conversion normally occurs from O-state to C-state upon binding of an anion to PS II. Fluoride appears to represent an exception, as binding was always associated with inhibition of oxygen evolution and almost identical inhibitory constants, 17 mM and 14.5 mM, respectively, were found, after short and long time incubation. The inhibitory action of fluoride observed here agrees with earlier observations of the effect of F^- in PS II preparations depleted of Cl^- using other methods (*22*, *23*). The addition of Cl^- to PS II membranes incubated with F^- for 3 h restored oxygen evolution instantaneously, which is consistent with earlier observations (*13*) of rapid restoration of the original distribution of the S_2 state EPR signals after addition of Cl^- to a sample incubated with fluoride. This is unexpected since the rate of dissociation of F^- in the absence of Cl^-, measured as the recovery of oxygen evolution activity, was found to be very slow ($t_{1/2} \approx 45$ min) in a glycerol-containing medium (Table 1).

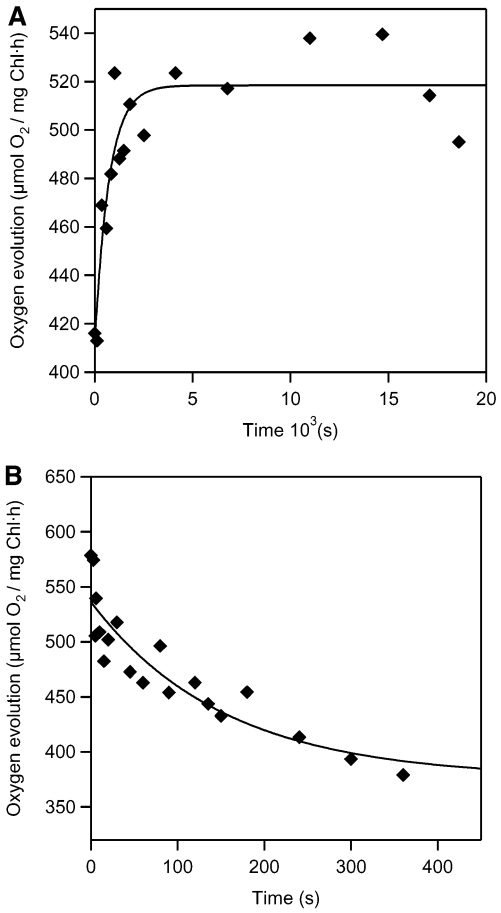

FIGURE 5: Kinetics of association and dissociation of Cl^- in PS II membranes. (A) Time-dependent rise in activity of Cl^--depleted PS II membranes incubated as indicated with 200 μM NaCl in 20 mM MES−NaOH, pH 6.3, and 1.4 M glycerol in darkness at 0 °C and assayed for activity 10 s after dilution in Cl^--free buffer in the oxygraph. (B) Time dependence for the dissociation of Cl^- from PS II membranes. PS II membranes were treated for 2 h in darkness at 0 °C with 200 μM NaCl in 20 mM MES−NaOH, pH 6.3, and 1.4 M glycerol. The dissociation of Cl^- was followed by varying the delay time as indicated between the dilution of the treated samples in Cl^--free buffer in the oxygraph chamber and the measurement of the oxygen evolution rate.

The rate of association of Cl^- (Figure 5A) was determined from the time-dependent increase of oxygen evolution activity in Cl^--depleted PS II membranes incubated for various times in a medium containing 200 μM Cl^-. The data,

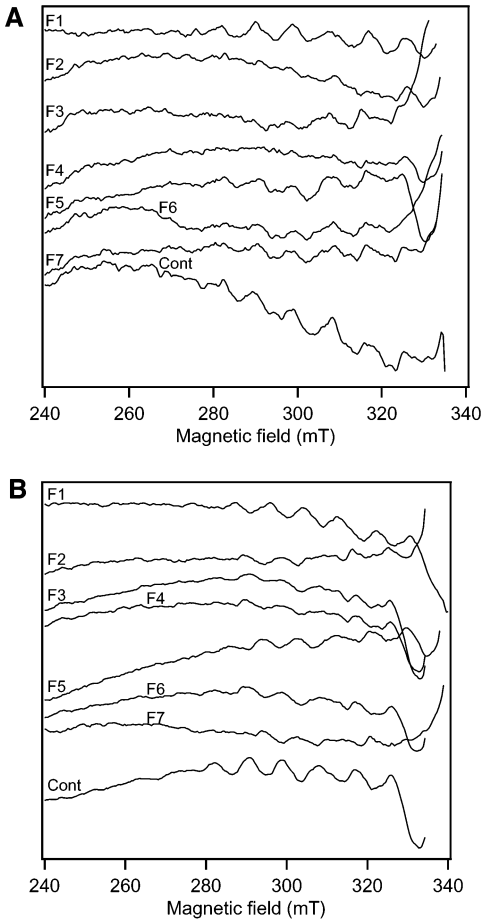

FIGURE 6: Amplitude of the S_2 state multiline signal in Cl^--depleted and Cl^--reconstituted PS II membranes illuminated by laser flashes. The amplitude of the signal was measured as the sum of amplitudes of the two strongest hyperfine (at 290 and 300 mT) lines on the low-field side of $g = 2$ in the spectrum. (A) Dark-adapted, Cl^--depleted PS II membranes in EPR tubes were illuminated by one to seven laser flashes (F1−F7) before freezing for EPR measurements. The multiline signal generated by continuous illumination (cont) at 200 K is shown for comparison. The figure shows the low-field part of the multiline signal as the difference after subtraction of the EPR signal from a dark-adapted sample. Sample preparation and conditions for EPR are as described in Materials and Methods. (B) Amplitude of the S_2 state multiline signal in Cl^--reconstituted PS II membranes illuminated by laser flashes. NaCl (10 mM) was added to Cl^--depleted PS II membranes before dark adaptation for 2.5 h at 0 °C.

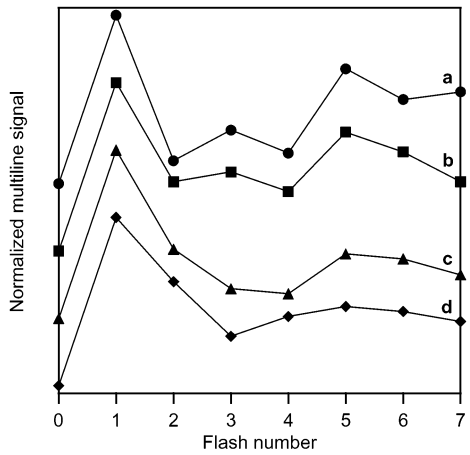

FIGURE 7: Flash-dependent oscillations in the amplitude of the multiline EPR signal in Cl^--depleted PS II membranes reconstituted with various anions. Traces a−d represent the amplitudes of the signal in (a) Cl^--depleted PS II membranes and depleted membranes reconstituted with 10 mM (b) NaCl, (c) $NaNO_3$, or (d) NaI. Conditions for sample preparation, laser illumination, and EPR are as in Figure 6. The traces have been displaced vertically for clarity.

fitted to a single-exponential curve, gave $t_{1/2} = 10$ min for the association of Cl^- in the C-state. This value, obtained in the presence of glycerol, is in close agreement with earlier determinations of the association rate constant for Cl^- in the C-state in a sucrose medium (*13*). In Figure 5B the decrease in oxygen evolution rate resulting from the dissociation of Cl^- from the C-state is shown. Analysis of the dissociation kinetics gave a value of $t_{1/2} = 50$ s. Thus, the conversion from the C-state to the O-state seems to be significantly faster in glycerol than in sucrose where the half-time for the dissociation of Cl^- in the high-affinity mode was $t_{1/2} \approx 1$ h (*12*).

EPR Studies of S-State Cycling in Cl^--Depleted and Anion-Reactivated PS II Membranes. Dark-adapted, Cl^--depleted PS II membranes, converted to the S_1 state (*17*) and then illuminated by a single laser flash, displayed a multiline EPR signal typical of the S_2 state (Figure 6A). The intensity of the signal was similar to that generated in depleted membranes after photoaccumulation of the S_2 state by continuous illumination at 200 K, a procedure which quantitatively converts S_1 centers to the S_2 state and thus gives an indication of the total number of active centers in the sample. The similar amplitudes of the S_2 multiline signals generated by the two different methods confirm that the flash given to the synchronized membranes in the S_1 state was saturating. Very little multiline signal could be detected in an identical sample illuminated by two flashes, which shows that virtually all centers in the S_2 state after the first flash proceeded to the S_3 state on the second flash. Additional flashes given to identical samples resulted in an damped oscillation in the amplitude of the multiline signal with maxima on the first and fifth flash (Figure 6A), as has been observed earlier with oxygen-evolving, Cl^--sufficient PS II preparations (*14*, *24*, *25*). Depleted centers, reconstituted with Cl^- by incubation with the anion for 2.5 h at saturating concentrations ($K_d = 13$ μM) (Figure 6B), which is long enough to convert the Cl^- binding site from a low-affinity site to one with a high affinity (*13*), displayed an identical behavior (Figure 6B). The somewhat lower amplitude of the multiline signal in the Cl^--depleted PS II centers compared to that in centers reconstituted with Cl^- is fully accounted for by the well-known depletion-induced shift in the relative intensities of the two S_2 state EPR signals in favor of the $g = 4.1$ signal (Figure 9, spectra a and b). The almost identical results obtained with Cl^--depleted and Cl^- -reconstituted membranes show that absence of Cl^- does not significantly reduce the number of centers which are able to cycle through the S-states. This result fully agrees with that obtained from the oxygen evolution experiments, which shows no loss of active centers after Cl^- depletion (Figure 3B) in addition to losses (about 20%) during the long dialysis required.

Among the anions investigated in addition to Cl^-, Br^-, I^-, and NO_3^- supported an oscillation of the multiline signal when a series of laser flashes were given to Cl^--depleted PS II membranes reconstituted with saturating concentrations of the anions (Figure 7). Br^-, which shows activation properties very similar to those of Cl^-, displayed an

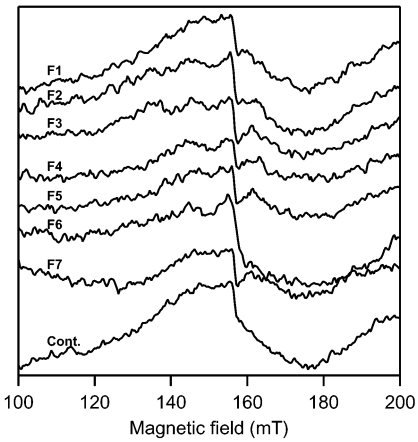

FIGURE 8: Amplitude of the S_2 state $g = 4.1$ EPR signal in Cl^--depleted PS II and fluoride-treated membranes illuminated by laser flashes. NaF (25 mM) was added to Cl^--depleted PS II membranes before dark adaptation for 2.5 h in darkness at 0 °C. Other conditions are as in Figure 6.

oscillation pattern almost identical to that of Cl^- (not shown). Also, the amplitudes of the signals were comparable for these anions. The S_2 state EPR spectrum of dialyzed, F^--treated ($K_d = 15$ mM) membranes was characterized by a very small multiline signal and a strong $g = 4.1$ signal (Figure 9B, spectrum f). The low amplitude of the multiline signal made a detailed analysis of its behavior after flash illumination difficult, but no evidence of oscillations in the field range of the signal could be detected. However, a large $g = 4.1$ signal was observed after the first flash which did not change much in amplitude on the following flashes (Figure 8). This indicates that F^- when replacing Cl^- in PS II allows formation of the S_2 state after a light flash as well as after continuous illumination at 200 K (Figure 9) (*13*). The bound fluoride prevents further S-state advancement of the OEC, which is fully consistent with the activity data obtained with dialyzed, F^--substituted PS II membranes (Table 1). These results represent another significant difference from what has been observed with F^--substituted PS II membranes depleted of Cl^- using a high-pH jump (*22*) where a period of four oscillations in the $g = 4.1$ were observed together with a reduced, although still significant oxygen-evolving activity. Our data clearly support a block of the S_2 to S_3 transition by fluoride in PS II membranes depleted of Cl^- by dialysis. The S-state turnover and residual activity observed by van Vliet and Rutherford (*22*) under similar conditions suggest incomplete Cl^- removal, which may taken to illustrate the variable efficiencies of the different depletion methods used.

The results from reactivation of Cl^--depleted membranes with NO_3^- may need a comment since a normal amplitude of the multiline signal after the first flash and a well-developed oscillation of the signal were observed despite the low restored activity. Nitrate has been observed to slow the oxygen-releasing S_3 to S_0 reaction in PS II membranes treated at alkaline pH with sulfate (*23*) and to reduce the stability of the higher oxidation states of the OEC. After depletion of Cl^- by dialysis, there was no large difference in the flash pattern of the multiline signal between NO_3^--treated and Cl^--reconstituted membranes illuminated by 1 Hz laser flashes (Figure 7). Thus, the stabilities of the S-states in PS II centers reconstituted with NO_3^- do not seem to differ much from those in Cl^--reactivated material, at least not in the seconds time regime.

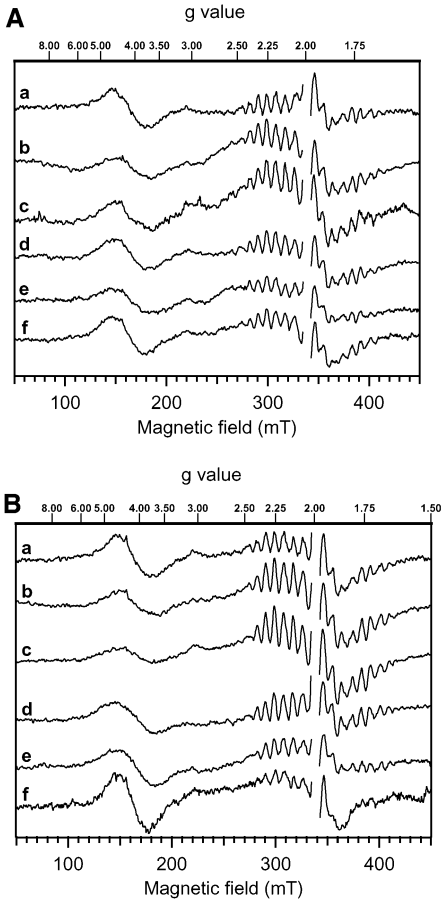

FIGURE 9: S_2 state EPR spectra of Cl^--depleted PS II membranes resupplied with various monovalent anions. (A) Spectra obtained after 20 s incubation with the anion: (a) Cl^- depleted; (b) 10 mM NaCl; (c) 10 mM NaBr; (d) 10 mM NaI; (e) 10 mM $NaNO_3$; (f) 10 mM NaF. (B) Spectra a−f as in (A) but after incubation for 2.5 h in darkness at 0 °C. The EPR spectra are presented as light minus dark difference spectra. Preparation of EPR samples and conditions for EPR are as described in Materials and Methods.

The S_2 State EPR Signals in the O- and C-States. To study the effects of anions on the EPR properties of the O- and C-states selectively, a solution with the appropriate anion was rapidly mixed with Cl^--depleted PS II membranes in an EPR tube and frozen at 200 K within 20 s in a mixture of dry ice and ethanol (O-state). Alternatively, the added anion was incubated with the depleted PS II membranes on ice in darkness for 2.5 h and rapidly frozen at 200 K (C-state). Generally, the equilibrium between the two EPR signals of the S_2 state, the multiline and the $g = 4.1$ signals, which are assumed to represent different spin states ($S = {}^1/_2$ and $S = {}^5/_2$ or $S = {}^3/_2$, respectively) of the manganese cluster (*26*, *27*), is shifted toward a small multiline and a large $g = 4.1$ signal in Cl^--depleted membranes (Figure 9, spectrum a). Addition of Cl^- at concentrations saturating the O-state restored the equilibrium toward a large multiline signal and a small $g = 4.1$ signal (Figure 9A, spectrum b). A barely observable further shift toward the multiline state was seen following the conversion of the O-state to the C-state (Figure 9B, spectrum b). The S_2 state multiline signal from membranes with bound anion in the O-state was sometimes

accompanied by a broad, underlying signal, which might originate from a broadened multiline signal.

Bromide, I^-, and NO_3^- induced a similar shift of the equilibrium between the EPR signals from the S_2 state as Cl^- when added to anion-depleted PS II membranes (Figure 9, spectra c−e) with only small further signal shifts on the O- to C-state conversion. The experiment with I^- demonstrates that no strong correlation exists between the relative intensities of the two S_2 state signals and the oxygen-evolving activity; the relative intensities of the two S_2 state signals were similar in the C- and O-states, respectively, while oxygen evolution was reactivated in the C-state but inhibited in the O-state (Figure 4). The addition of F^- to depleted membranes shifted the equilibrium toward a large $g = 4.1$ signal and a small multiline signal, and the effect was more pronounced in samples incubated for 2−3 h (Figure 9, spectrum f).

DISCUSSION

Numerous earlier studies have indicated that Cl^- is necessary for oxygen evolution. However, our previous (*12*, *13*) and present results show that water oxidation can be maintained at a high level by photosystem II even in the absence of Cl^-. With glycerol as a cryoprotectant the dialyzed, Cl^--depleted PS II membranes show an oxygen evolution rate, extrapolated to saturating illumination, around 500 μmol of O_2 (mg of Chl h)$^{-1}$, corresponding to about 55% of the control rate compared to about 30% in sucrose (*12*, *13*). In agreement with our earlier studies using sucrose media, the residual oxygen evolution activity in Cl^--depleted PS II membranes in a glycerol-containing buffer cannot be explained as the activity of a fraction of PS II centers containing Cl^-. First, PS II does not contain nonexchangeable Cl^-, and Cl^- introduced during culture is slowly lost from the isolated PS II membranes in a Cl^--free medium with a rate comparable to the loss of Cl^- from PS II membranes labeled after preparation (*28*). A later repetition of the culture-labeling experiment showed that no labeled Cl^- was bound after 22 h of dialysis of the isolated PS II membranes (initially about 0.5 Cl^-/PS II) against a Cl^--free buffer. With a half-time for the dissociation of Cl^- of about 1 h (*15*) or even less in glycerol (Table 1), the dialysis procedure used is expected to remove all initially bound Cl^-. Second, contaminating chloride in the medium cannot account for the residual activity. With a K_d of 13 μM for reactivation, less than 1 μM contaminating Cl^- means that less than 7% of the centers have associated Cl^-. Perhaps the strongest arguments for a Cl^- -independent activity can be found from plots of the light dependence of the oxygen evolution rate (Figure 3B). If Cl^- was necessary for oxygen evolution, its removal should result in a decrease in the quantum yield of the reaction, equivalent to a decrease in the intercept on the abscissa axis in Figure 3B. Instead, the plots show a common point of interception on the abscissa axis for depleted and reactivated membranes, which confirms our earlier sucrose data (*13*). Furthermore, the similar amplitudes of the oscillating multiline EPR signal in Cl^--depleted and reconstituted PS II membranes (Figure 6) also show that all depleted centers participate in the flash-induced cycling of the S-states. These results clearly indicate that the OEC in PS II membranes Cl^--depleted by dialysis turns over as a response to illumination in a manner which does not significantly differ from the behavior of Cl^--sufficient membranes. The reduced activity may result from a retardation of one the S-state transitions, possibly S_2 to S_3, which is known to be sensitive to perturbations (see below). It should be added that the rate limitation in dialyzed PS II membranes does not seem to be associated with the acceptor side since studies of the decay of the induced fluorescence show that the electron-transfer steps here are unaffected by Cl^- depletion (K. Olesen, unpublished results).

One obvious reason for the existing confusion regarding the requirement for Cl^- in the water-splitting mechanism is that commonly used Cl^--depletion methods have been optimized to eliminate oxygen evolution with little regard to the effects on the integrity of the PS II membranes. The commonly practiced procedure, involving SO_4^{2-} at alkaline pH, has a clear effect on the polypeptide composition of PS II membranes (*20*, *23*, *29*) particularly in combination with sucrose (Figure 1). Removal of the 17 and 23 kDa extrinsic proteins, which play an important role preserving the high-affinity site for Cl^- in PS II membranes (*12*), is known to lead to a complete loss of oxygen evolution, which can be restored by high concentrations of Cl^- (*5*) binding to a single, low-affinity binding site (*20*, *23*). In contrast, the polypeptide pattern of PS II membranes after Cl^- depletion using dialysis reveals no loss of proteins compared to untreated membranes (Figure 1), Therefore, the observed Cl^- requirement of a PS II preparation, which has been depleted of Cl^- using SO_4^{2-} at elevated pH or related methods, may have resulted as a consequence of the tendency of these methods to irreversibly alter PS II centers (*10*). In such PS II membranes, further advancement of the S_2 state is inhibited (*3*, *22*, *30*).

Our present and previous (*12*, *13*) data are consistent with two binding modes for Cl^- and related anions, characterized by weak binding of a rapidly exchanging anion to a site in an open, accessible state (O-state) and slow conversion to a less accessible or closed, high-affinity state (C-state), although two separate, mutually exclusive sites cannot be excluded. As seen in Figure 9, spectrum b, there is only a small difference in the distribution in intensity of the two S_2 state EPR signals with Cl^- bound in the O-state (short incubation with Cl^-) and the C-state (long incubation). That and the similar water oxidation activities (Table 1, Figure 2) suggest that the environment around the manganese cluster is essentially the same in the two states.

Among other anions examined, Br^- appeared to behave as Cl^- with an anion-induced reactivation of the depleted PS II membranes associated with a change from O-state to C-state binding (Figure 2B, Table 1) in agreement with earlier work from this laboratory (*13*). In contrast, I^- is initially inhibiting in the low-affinity mode (O-state) (Table 1, Figure 4), switching to activating as conversion to the tight-binding C-state occurs. A comparison of the tightly bound I^- with Cl^- reveals similar properties with respect to binding affinity and kinetics of binding and dissociation while the recovery of activity is somewhat lower with I^- than with Cl^- (Table 1). Strangely, the inhibitory action of I^- in the O-state is not clearly reflected in the EPR properties of the S_2 state. Addition of I^- to the anion-depleted membranes immediately shifted the intensity of the two signals back roughly to the distribution seen with Cl^- (Figure 9, spectrum d) in contrast to the result with the inhibitory F^- (see below),

indicating that iodide does not perturb the manganese center significantly, not even when it inhibits in the O-state.

Low recovery of oxygen evolution by I^- has been observed before (*6*, *31*) and has been ascribed to iodination of an essential group, identified as $TyrZ^{ox}$, by the I_3^- formed from the oxidation of I^- (*32*, *33*). The lower stability of higher oxidation states of the OEC and the slower S_3 to S_0 transition in polypeptide-depleted membranes (*23*) have also been suggested to contribute to the low activity. The O-state inhibition by I^- seen in dialyzed PS II membranes may indicate that the location of the binding site in this state is close to the tyrosine. The change in binding mode from O-state to C-state might be associated with a conformational change, which makes the I^- ion inaccessible for oxidation. Alternatively, the reduction of the strong oxidants $P680^+$ and/or $TyrZ^{ox}$ by electrons from the OEC, which is normally in the nanosecond and microsecond range, respectively (*34*, *35*), is rapid enough in the C-state to compete efficiently with I^-. The O-state to C-state conversion appears to eliminate the inhibitory binding site altogether, as addition of 10 mM I^- to membranes with I^- bound in the C-state failed to inhibit oxygen evolution activity, supporting the idea that anion binding generally occurs at a common site (however, cf. F^- below). After Cl^- depletion by a transient high-pH jump, I^- bound to the Cl^- site did not seem to be accessible for oxidation in the S_2 state (*36*) as with I^- bound in the C-state in dialyzed PS II membranes. Contributions other than iodination may contribute to inhibition by I^- in the O-state as also Cl^-, although at much higher concentrations, inhibits oxygen evolution. Presently, we have no explanation for the latter effect although nonspecific binding may be involved.

There is evidence of a separate site, not shielded by the extrinsic polypeptides, where I^- apparently was observed to deactivate the higher oxidation states by reduction (*23*). It is not clear if the latter site is related to the inhibitory I^- site in the O-state in dialyzed membranes since the observed destabilization of the higher S-states was not considered to be responsible for the inhibition of steady-state oxygen evolution.

Nitrate appears to behave essentially as Cl^- and Br^-, reacting as expected from the one-site, two-state model, although the low level of reactivation made it difficult to estimate affinity properties and kinetic constants of binding and dissociation with precision. Although binding of nitrate in the C-state after extended incubation was weaker than observed immediately after addition of the anion and much weaker than that of Cl^-, the ion followed the general trend of restoring the original (Cl^--dependent) distribution of the S_2 state EPR signals (Figure 9, spectrum e). The lack of immediate effect of Cl^-, when added to a sample preincubated with NO_3^-, suggests that NO_3^- and Cl^- compete for the same site (*12*) and that conversion to the O-state precedes exchange. Evidence of competition was also deduced from optical changes associated with the S-state transitions (*11*).

Fluoride appears to be incapable of inducing a conversion from the O- to C-state as membranes, treated for several hours with the anion, recovered their activity within seconds after addition of Cl^-, which is expected to replace F^- by virtue of its stronger binding in the O-state. Unexpectedly, the dissociation of F^- was slow in the absence of competing Cl^- (Table 1). The acceleration of F^- release by competing Cl^- is similar to the effect of NO_3^- on the release of Cl^- in SO_4^{2-}-treated PS II membranes (*11*) where the exchange was suggested to take place in a sequestered domain in PS II through a large diffusion barrier. Alternatively, inhibition by F^- may occur from a remote site and that Cl^- by binding somehow induces a change in its affinity properties.

The difference between anions capable of inducing the C-state and F^- is underlined by the behavior of the S_2 state EPR signals after flash illumination. Chloride, Br^-, I^-, and NO_3^- all supported similar oscillations of the multiline signal as a response to laser flashes (Figure 7, results from Br^- not shown). Obviously, effects on the S-state transitions or the stabilities of the S-states, responsible for the differences in steady-state activities supported by these ions, are not large enough to be readily detected in the flash pattern. Similar conclusions were arrived at from optical studies of polypeptide-depleted PS II membranes (*23*).

Chloride has been considered to be located close to or even directly coordinated to the manganese cluster (for a recent review, see ref *37*). A close association with the manganese cluster is supported by the behavior of the EPR signals from the S_2 state, which generally show a shift toward higher intensity if the $g = 4.1$ signal at the expense of the multiline signal is absent of Cl^- (Figure 9, spectra a and b). Results from EXAFS with F^- have also indicated a close association with manganese (*38*). However, as noted above, F^- may bind in a manner deviating from that of the other anions.

A close involvement of Cl^- with manganese in the mechanism of oxygen evolution is difficult to conceive for several reasons. First, and most importantly, high levels of activity can be maintained in the absence of Cl^-. Second, the nature of the activating anion does not seem to be very important for the reaction except for fluoride. A realistic model for Cl^- involvement must take these observations into account as well as it should indicate how a Cl^- dependence may arise from perturbations in the PS II structure.

There are several independent observations, which may help to clarify the role of Cl^- in the water oxidation mechanism. Labeling experiments with ^{36}Cl have shown that binding of Cl^- in the C-state is highly pH dependent (*12*) with a steep decline in binding efficiency at pH values above a pK_a of 7.5 for an unidentified group controlling the retention of Cl^-. Dilley and co-workers (*39*) observed a close connection between the binding of Cl^- and the protonation state of proteins in PS II, suggesting a possible involvement of Cl^- in a proton-sequestering domain connecting the water-oxidizing system and the coupling factor. They also found a set of protons associated with lysines with an anomalously low pK_a value around 7.5 (*40*), similar to that involved in Cl^- binding in dialyzed membranes. Furthermore, the deprotonation of the lysines was surprisingly slow, with a half-time in the order of 1 h, in close agreement with the half-time for the dissociation of Cl^- from PS II in the closed state (*13*). These observations taken together suggest that Cl^- may participate in a structure involving also the low pK_a lysines, which controls the overall rate of water oxidation.

Since the manganese cluster, where the oxidation of substrate water takes place, is buried close to TyrZ of the D1 subunit and protected by the 33 kDa (PsbO) protein from the outside (*41*, *42*), there should be an exit pathway for released protons in analogy with the specific proton channel

leading to the binuclear metal center in cytochrome *c* oxidase (*43*, *44*). A similar channel in PS II should contain protonable groups, i.e., amine and carboxylic amino acid side chains. These groups together with Cl^- and bound water molecules may form a relay system which permits the transport and release of protons from water oxidation to the bulk phase, in analogy with the suggestions by Theg and Homann (*9*) and Dilley and co-workers (*39*, *45*), although we would prefer to assign the role of Cl^- in such a network specifically to PS II and water oxidation. The apparent close relation between Cl^- binding and the unusual protonation properties of the lysines implies that the integrity of the proton-relay network should depend on trapped Cl^-. We propose that release of Cl^- from PS II, induced either by a slow conversion from the C- to O-state (*12*) or by an alkaline treatment (*46*), disrupts the normal proton-relay network and retards the release of protons from the water-splitting reaction. The lower but still considerable residual activity in the O-state of dialyzed PS II membranes suggests that an altered, Cl^--independent but less efficient proton-relay system results. A normal proton-relay network is restored on the O- to C-state conversion, induced by rebinding of Cl^-. As concentrations of Cl^- around 1 mM are able to raise the activity of the O-state to normal levels, an efficient, alternative Cl^--dependent charge-relay system appears to be formed.

The larger perturbation of the "O-state" resulting from high pH−sulfate treatment is underlined by the loss of extrinsic proteins (Figure 1). The complete loss of activity can be accounted for if loss or severe perturbation of the proteins completely destroys the proton network. The restoration by high concentrations, i.e., several millimolar, of Cl^- (*19*) implies that an alternative proton release pathway can be established also in this case.

Our interpretation of the anion requirement in PS II is that the monovalent anions function by providing a negative charge, not to compensate for positive charges formed in the water oxidation reaction but for the formation and maintenance of a charge-relay network involving amino acid side chains. This may explain the less stringent anion requirement but also why F^- inhibits water oxidation. F^- is able to participate in hydrogen-bonded structures with other groups and may induce the formation and breaking of hydrogen bonds which prevents the normal transfer of protons from the water-splitting reaction. The slow inherent dissociation of fluoride suggests that the ion becomes integrated in this structure. Since Cl^- is able to rapidly eliminate the F^--induced inhibition, a reorganization appears to take place with release of fluoride and restoration of the normal proton-release pathway.

In summary, results in this and in our earlier studies have shown that photosynthetic water oxidation in the absence of Cl^- may occur at significant rates, up to about 50% of that observed in the presence of Cl^-. The dialysis depletion method for Cl^- depletion used in our laboratory does not release extrinsic proteins in contrast to methods involving high pH and SO_4^{2-}, where such perturbations may induce a Cl^- requirement (*10*). Chloride and several other monovalent anions which are able to support high rates of water oxidation in dialysis-depleted PS II membranes transform PS II from an open state with weak binding to a closed state with tight binding of the anion. We propose that Cl^- together with lysine side chains and other charged amino acids maintains a proton-relay network, which allows the transport and release of protons from the water-splitting reaction (see also ref *47*). Different methods of Cl^- depletion may cause a more or less severe perturbation of this network with complete or partial loss of oxygen-evolving activity as a result.

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BI026175Y