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A One-Site, Two-State Model for the Binding of Anions in Photosystem II[†]

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ABSTRACT: Photosystem II membranes, dialyzed against a Cl^--free buffer to remove bound Cl^-, lost about 65% of the control activity. A light-intensity study of the Cl^--free membranes showed that all PS II centers were able to evolve oxygen at about 35% of the control rate when measured in Cl^--free medium. The Cl^--depleted membranes were immediately (<15 s) reactivated to 85−90% of the original activity by the addition of fairly high concentrations of Cl^- ($K_d = 0.5$ mM), but both Cl^- and the activity were promptly lost when the membranes immediately after reactivation were diluted in a Cl^--free medium. However, stabilization of Cl^--binding could be accomplished by prolonged incubation in the presence of Cl^-. The transition to stable binding, followed using $^{36}Cl^-$, occurred over several minutes. The stable binding was further characterized by a K_d of 20 μM and a $t_{1/2}$ for dissociation of about 1h [Lindberg et al. (1993) *Photosynth. Res. 38,* 401−408]. The effects on S_2 signals of removal of Cl^- were studied using EPR. The depletion of Cl^- was accompanied by a shift in intensity toward the $g = 4.1$ signal at the expense of the multiline signal. When Cl^- or Br^- but not F^- was added to the depleted PS II membranes, the original distribution of the signals was immediately (<30 s) restored. We propose that Cl^--binding responsible for high oxygen-evolution activity and normal EPR properties of the S_2 state may occur either as high affinity ($K_d = 20$ μM) and slowly exchanging ($t_{1/2} = 1$ h), or as low affinity ($K_d = 0.5$ mM) and rapidly exchanging ($t_{1/2} < 15$ s). Our results suggest that Br^- but not F^- has a mode of binding similar to that of Cl^-. The high-affinity state is the normal state of binding, but once Cl^- has been removed, it will first rebind as low-affinity, rapidly exchanging followed by conversion into a high-affinity, slowly exchanging mode of binding.

It has long been thought that Cl^- plays an essential role in the oxygen-evolving process [reviewed in Wydrzynski et al. (1990), Rutherford et al. (1992), and Yocum (1992)], although there is a lack in consensus on where and how Cl^- ions specifically interact with the water-oxidizing site. The loss of the two polypeptides, 16 kDa and 24 kDa, induces an increased demand for Cl^- in order to retain optimal function of the water-oxidizing reaction. This may suggest a role for Cl^- in maintaining the protein organization needed for O_2-evolution (Miyao & Murata, 1985; Homann, 1988a,b).

Cl^--depletion experiments have been reported to result in inhibited formation of S_2 (Itoh et al., 1984; Theg et al., 1984) or the formation of a modified S_2 state (Ono et al., 1986), while other groups suggest that the block occurs after the formation of S_3 (Baumgarten et al., 1990; Boussac et al., 1992). Evidence from these studies in addition to results from Cl^-/NH_3 competition experiments (Sandusky & Yocum, 1984) and X-ray absorption studies (Yachandra et al., 1991) have suggested models in which the Cl^- ion may be ligated to Mn itself. Previous studies on Cl^- have often involved treatments such as incubation in relatively high concentrations of Na_2SO_4 (Itoh & Uwano, 1986; Ono et al., 1986) or uncouplers and/or high pH (Kelly & Izawa, 1978, 1979; Theg & Homann, 1982; Homann, 1985, 1988a) in Cl^--free medium. Such conditions are assumed to cause the displacement of Cl^-. In Lindberg et al. (1990) PS II[1] membranes isolated from spinach grown on medium containing $Na^{36}Cl$ were found to have a binding site for one Cl^- ion that is in slow exchange with the surrounding medium. This tightly bound Cl^-, which was found to have a dissociation constant of around 20 μM and to be very sensitive to the presence of the three extrinsic polypeptides, 17, 24, and 33 kDa, could be removed by dialyzing the PS II membranes against Cl^--free medium for 15−20 h. After dialysis the Cl^--free membranes had an oxygen-evolution activity of 30−40% compared to membranes incubated with Cl^- (Lindberg et al., 1993). In order to further characterize the binding of anions in PS II we have studied the effects on O_2−evolution and S_2 state EPR signals (multiline and $g = 4.1$) after addition of Cl^-, Br^-, or F^-.

MATERIALS AND METHODS

Sample Preparation. Photosystem II membranes (250 chlorophyll/PS II) were prepared from spinach as described in Franzén et al. (1986). The oxygen-evolution activity of the PS II preparations at saturating light intensities was about 500 μmol of O_2/(mg of chlorophyll h). Cl^--free PS II membranes were prepared by suspending 10−20 mg of Chl of the PS II membranes (1 mL pellet) in 35 mL of Cl^--free buffer, 400 mM sucrose (BDH ARISTAR, <0.5 ppm Cl^-), and 20 mM Mes-NaOH, pH 6.3 (Sigma Ultra, >0.005% Cl^-) as described in Lindberg et al. (1993) followed by centrifu-

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[1] Abbreviations: Chl, chlorophyll; PS II, photosystem II; MES, 2-(*N*-morpholino)ethanesulfonic acid; EPR, electron paramagnetic resonance; PPBQ, phenyl-*p*-benzoquinone.

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Table 1: Retention of Cl^- in Freshly Prepared and Dialyzed PS II Membranes Isolated from Spinach Grown on $Na^{36}Cl$ before and after Dialysis[a]

sample	Cl^-/PS II[d]
PS II membranes before dialysis[b]	0.45
PS II membranes after dialysis[c]	0.003

[a] Dialysis against 400 mM sucrose, 20 mM MES-NaOH (pH 6.3), 15 mM NaCl, 5 mM $MgCl_2$. [b] Sample taken 1.75 h after the addition of Triton X-100 to the thylakoids. [c] Dialysis for 18 h on ice in the dark. [d] Chloride remaining from original content.

gation at 12 100*g* for 2.5 min, and repeating this washing procedure three times. This removes the unspecifically bound Cl^- (washed membranes). The membranes were further dialyzed against Cl^--free buffer for 15−20 h in darkness at 0 °C in order to remove the slowly exchanging Cl^- (dialyzed membranes).

^{36}Cl-labeled PS II membranes were prepared by suspending washed membranes in buffer containing NaCl (42% $Na^{36}Cl$). Labeled samples were washed and measured as described in Lindberg et al. (1990, 1993).

Oxygen-Evolution Activity Measurements. Oxygen-evolution was measured with a Clark-type electrode (Hansatech) as described by Andréasson et al. (1988) in Cl^--free buffer and 1 mM PPBQ with further additions as indicated in figure legends.

EPR. X-band EPR signals from the S_2 state, induced by illumination of dark-adapted PS II samples at 200 K, were measured at a microwave power of 20 mW and a temperature of 11 K (Andréasson et al., 1988).

RESULTS

Cl^--Binding and O_2-Evolution

In previous experiments (Lindberg et al., 1990, 1993) we have demonstrated that PS II membranes isolated from spinach grown on medium containing $Na^{36}Cl$ retained about 1 Cl^-/PS II. Table 1 shows that the freshly prepared PS II membranes still retain significant amounts of the radioactive label, corresponding to 0.45 Cl^-/PS II. Since the remaining radioactivity is not permanently associated with the PS II membranes but can be removed by dialysis of the membranes for an extended period against a Cl^--free buffer, a fraction of the original radioactivity must have been lost during the preparation procedure, most likely by exchange with Cl^- in the surrounding medium. An approximate value of about 1−1.5 Cl^-/PS II for the original content can be calculated from the preparation time and remainder of the original Cl^- content with the assumption of simple dissociation kinetics with a half-time of 1 h (Lindberg et al., 1993). This value agrees well with the chloride content in freshly prepared, washed chloroplasts, which was determined to about 1/PS II in a separate experiment.

The dialyzed, Cl^--free PS II membranes still retain about 30−40% of their water-oxidizing activity when assayed in Cl^--free medium compared to a control, which has been dialyzed and assayed in buffer with 25 mM Cl^- (Lindberg et al., 1993). Similar results were found by Yachandra et al. (1986), who reported a 40% loss in O_2-evolving activity after repeated washing and dialysis against Cl^--free buffers. The residual activity was interpreted as an effect of incomplete removal of Cl^- as they were not able to prepare PS II particles unable to evolve oxygen by this method. The loss of activity is not permanent, however, as the water-oxidizing activity can be regained after the addition of Cl^-. As we observed that Cl^- depletion resulted in an increased sensitivity of the PS II membranes to inactivation by illumination at high light intensities, the activity measurements were carried out at non-saturating light conditions, hence the apparent low values for the water-oxidation activities. Thus, the question arises whether this loss of O_2-evolving activity during the dialysis is due to a total inactivation of 60−70% of the centers or a deactivation of all the centers to a 30−40% level of activity. In Figure 1 the water-oxidizing activities of the untreated, dialyzed, and reactivated PS II membranes, respectively, were measured at different

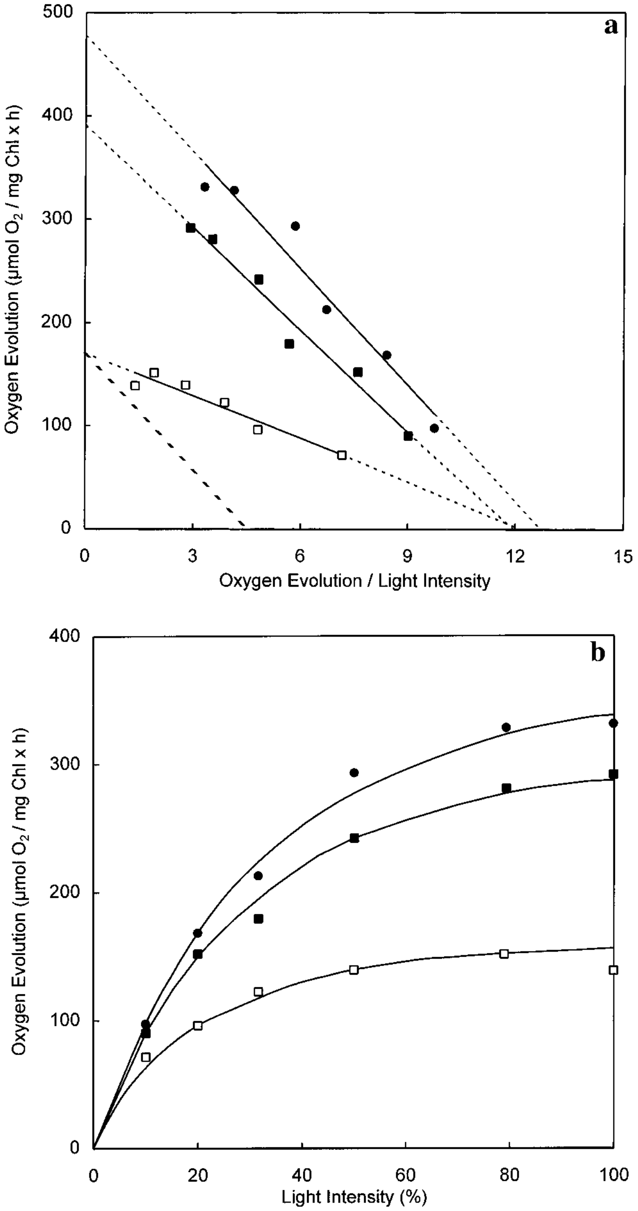

FIGURE 1: Plot of the oxygen-evolution activity against the activity divided by light intensity for dialyzed membranes (□), dialyzed membranes measured after readdition in Cl^- (■) and untreated (●) PS II membranes. The experiment was performed as described in Materials and Methods at identical chlorophyll concentrations with 25 mM NaCl included in the buffer for the untreated and reactivated samples. Samples were incubated for 15 s in the in the O_2-measuring cell before the light was turned on. The light intensity was varied with neutral density filters. The dashed line (- - -) shows a situation in which 35 % of the centers would be fully active. (b) Plot of activity versus light intensity.

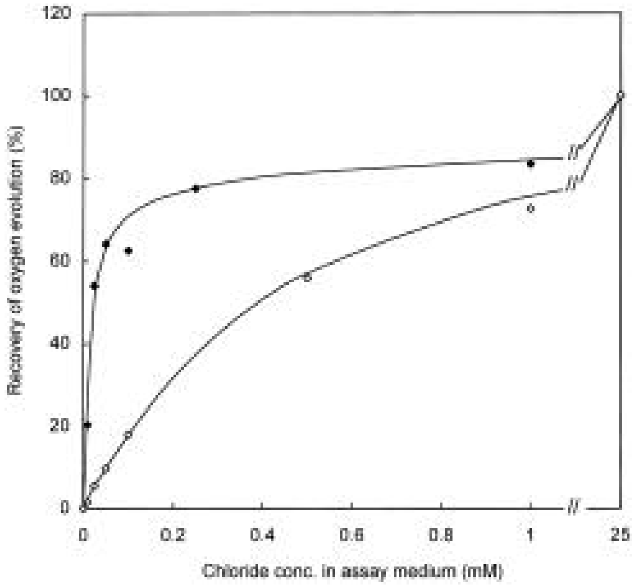

FIGURE 2: The Cl^--concentration dependent recovery of O_2-evolution activity in dialyzed and washed PS II membranes. (◆) PS II were washed twice in Cl^--free buffer, suspended for 2 h in buffer with 0−25 mM Cl^- included, and then assayed for O_2-evolution activity in the same medium. The oxygen-evolution activities at 0 and 25 mM Cl^- were 225 and 377 μmol of O_2/(mg of chlorophyll h), respectively. (◇) Washed PS II membranes were dialyzed against Cl^--free medium for 5 h and then assayed for oxygen-evolution activity in 0−25 mM Cl^- after incubation for 15 s in the O_2-measuring cell before the light was turned on. The oxygen-evolution activities at 0 and 25 mM Cl^- were 140 and 396 μmol of O_2/(mg chlorophyll h), respectively.

light intensities. When the activities were plotted against the activity divided by light intensity, linear plots were obtained. If the plots are analyzed in a way similar to Michaelis−Menten enzyme kinetics, the slope, the abscissa, and the ordinate intercepts of the lines correspond to the apparent Michaelis constant ($-K_m$), relative quantum yield, and the maximum rate for oxygen-evolution (V_{max}), respectively (Miyao & Murata, 1983; Franzén et al., 1986). The intercepts on the abscissa for the dialyzed membranes and untreated PS II membranes were very similar. This indicates that nearly 100% of the centers remained active after the dialysis treatment. However, the oxygen-evolving capacity per center in the Cl^--free membranes was only about 35% compared to the untreated membranes and was reactivated to about 85% of the original activity when resupplied with 25 mM NaCl. For comparison, the dashed line in Figure 1 shows the result expected in a case where 35% of the centers would be fully active and the remaining completely inactive.

The concentration of Cl^- required to reactivate the O_2-evolution activity completely in dialyzed membranes was investigated in Figure 2. PS II membranes, dialyzed for 5 h to remove most of the bound Cl^- [>85% depleted, see Lindberg et al. 1993] were suspended at concentrations of 0−25 mM Cl^- in the O_2-measuring cell for 15 s before the light was turned on. The activity could be rapidly recovered but only after the addition of fairly high concentrations of Cl^- ($K_d = 0.5$ mM). Thus, there seems to exists a site with low affinity for Cl^-, involved in the reactivation of oxygen-evolution. This should be compared with the amount of Cl^- needed to sustain high oxygen-evolving activity in PS II membranes equilibrated with Cl^- for longer times (also Figure 2). PS II membranes were equilibrated for 2 h at different concentrations of Cl^- and assayed for O_2-evolution activity at the same concentrations. Reactivation by Cl^- was accomplished by binding to a site with a K_d of about 20 μM. This value is identical to that earlier observed for slowly exchanging Cl^-. Thus, there seems to exist two types of binding for Cl^- in PS II, one caracterized by high affinity and slow exchange and another by low affinity and rapid exchange.

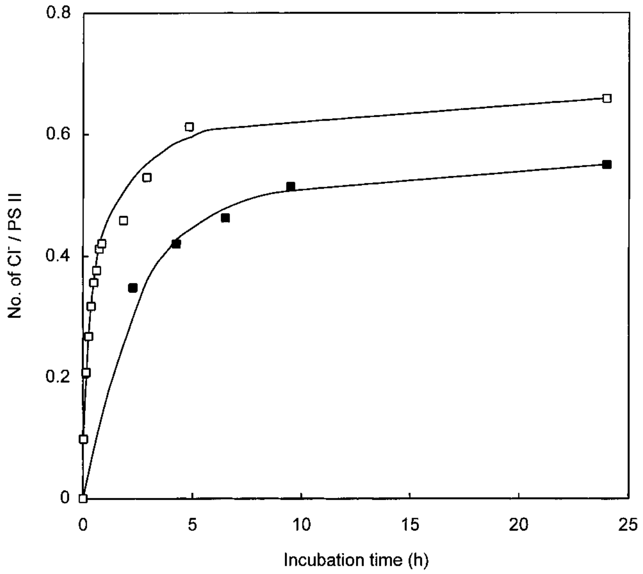

FIGURE 3: Binding of $^{36}Cl^-$ in PS II. Dialyzed (□) and washed (■) PS II membranes were incubated at 100 μM Cl^- (42% $Na^{36}Cl$). Samples were washed with Cl^--free buffer to remove unbound Cl^-, and the amount of bound $^{36}Cl^-$ was determined as in Lindberg et al. (1990).

To investigate the relation of the two modes of binding of Cl^- and reactivation with Cl^- further and especially if interconversion between them occurs, the difference in binding of Cl^- to dialyzed PS II membranes was investigated using labeling with $^{36}Cl^-$. With these membranes, which are initially in the low-affinity mode of binding, the time dependence for trapping of slowly exchanging Cl^- can be used to measure the rate by which the membranes are converted from the low- to the high-affinity binding. Furthermore, the binding takes place at an empty site and is not limited by the exchange with Cl^- already present in the site. From the data in Figure 3 one can estimate the half-time for interconversion to about 10 min at 100 μM added Cl^-. This should be compared to the binding of Cl^- in washed membranes where the labeling can be viewed as an exchange reaction between Cl^- present in the site and $^{36}Cl^-$ added in the medium (Figure 3). The rate of labeling is limited by the slow dissociation of Cl^- from PS II as shown in Lindberg et al. (1993). This is supported by the identical half-times of about 1 h for both binding at saturating concentrations of Cl^- and dissociation of Cl^- in washed membranes [also in Lindberg et al. (1993)].

The rate of conversion from low-affinity to high-affinity binding was also determined from the time-dependence of the reactivation of oxygen-evolution in dialyzed membranes incubated in the presence of 100 μM Cl^- (Figure 4a) or 2 mM Cl^- (Figure 4b). For total activation involving both types of Cl^--binding (high- and low-affinity), the oxygen-evolution activity was measured in an assay medium with 100 μM or 2 mM of Cl^-, respectively. To measure the activation due to high-affinity binding only, the PS II membranes were suspended in a Cl^--free medium and

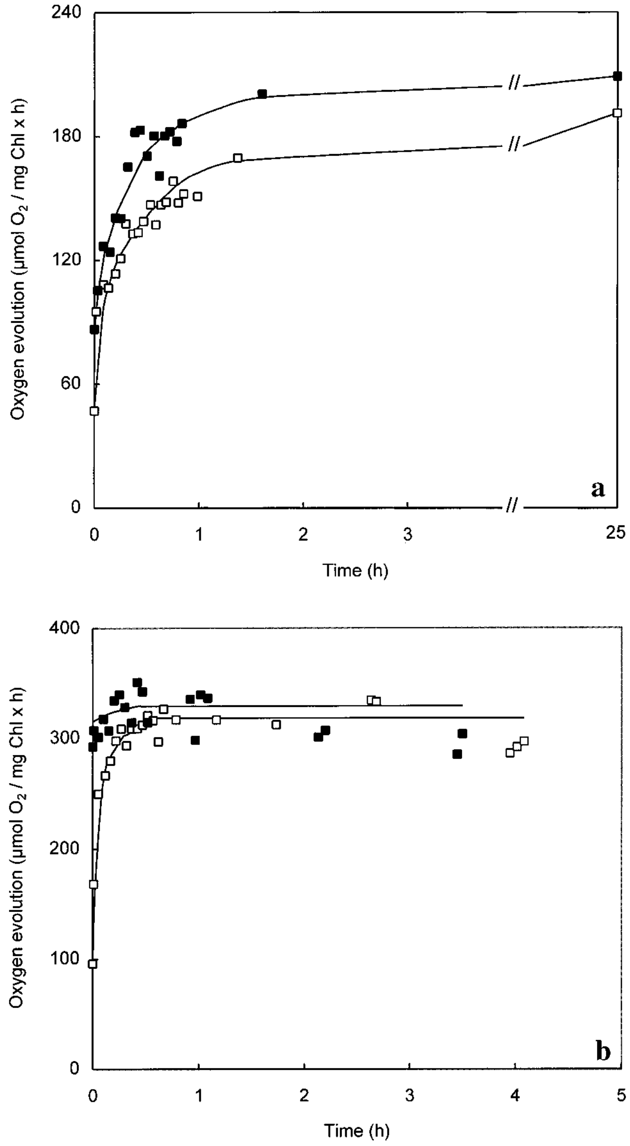

FIGURE 4: The reactivation of oxygen-evolution activity in dialyzed PS II membranes after incubation in (a) 100 μM NaCl and (b) 2 mM NaCl. (a) Oxygen-evolution activities in Cl^--free buffer (□) and in buffer with 100 μM Cl^- (■), respectively, were determined. Samples were incubated for 15 s in the O_2-measuring cell before the light was turned on. (b) Cl^--free PS II membranes were incubated in 2 mM NaCl, and the oxygen-evolution activity was measured in Cl^--free buffer (□) and in buffer with 2 mM Cl^- (■).

assayed. The total concentration of Cl^- in the measuring cell after dilution of the membranes was <1 μM for the membranes incubated in 100 μM Cl^- and <10 μM for the membranes in 2 mM Cl^-. With a dissociation constant of 0.5 mM for the rapidly exchanging, low-affinity Cl^-, this Cl^- should be released immediately on dilution in the Cl^--free medium. The high-affinity, slowly exchanging Cl^-, on the other hand ($K_d = 20$ μM and $t_{1/2} = 1$ h), should remain bound during the short time of the assay due to its slow dissociation, although the concentration of Cl^- is below 20 μM. If a slow conversion from low- to high-affinity occurs as a result of Cl^--binding, one would expect the relative difference between O_2-evolution activity in the Cl^--free and in the Cl^--containing medium to decrease with the time of pre-incubation in the presence of Cl^-.

With the membranes incubated in 100 μM Cl^- medium (Figure 4a) the progress of reactivation could clearly be resolved in both assay media, but the activity assayed in 100 μM Cl^- was higher than in the Cl^--free medium. The low activity observed in Cl^--free medium at the start of the incubation is the residual activity in the dialyzed membranes as Cl^- is not yet bound. The subsequent increase in activity closely follows the binding of high-affinity Cl^- as can be seen by comparison with Figure 3. The additional activity seen in the presence of 100 μM Cl^- in the assay medium at the start of incubation results from low-affinity binding of Cl^- in a fraction of the centers, but since the concentration of Cl^- is below the K_d (0.5 mM) for the low-affinity Cl^-, this site is not saturated and consequently full O_2-evolution activity is not reached. As the incubation continues, the high-affinity binding of Cl^- predominates and the relative difference in activity between the two assay media decreases, in accordance with our hypothesis.

For the membranes incubated in 2 mM Cl^- (Figure 4b) almost full reactivation was reached immediately when the activity was assayed in 2 mM Cl^-. Our explanation for this is that in this assay medium the concentration of Cl^- is well above the K_d of 0.5 mM determined for the rapidly exchanging Cl^-. Thus, the low-affinity binding for Cl^- dominates, giving high activity from the beginning. The lower reactivation observed in the Cl^--free assay medium results from the fast dissociation of low-affinity Cl^-, leaving only the influence from the fraction of centers with high-affinity binding of Cl^-. With longer incubation times in medium containing Cl^-, the difference in oxygen-evolution rates becomes negligible, indicating that Cl^- is no longer able to dissociate on dilution in the Cl^--free medium; i.e., the conversion from low-affinity to high-affinity binding is complete.

The restoration of activity when measured in Cl^--free assay medium was faster for the membranes incubated in 2 mM ($t_{1/2} = 6$ min) than for those incubated in 100 μM Cl^- ($t_{1/2} = 10$ min), showing a concentration dependence of the rate for the rebinding of high-affinity Cl^- which is close to saturation.

Effects of Cl^--Binding on S_2 State EPR Signals

The relative amounts of multiline and $g = 4.1$ signals have earlier been shown to be very sensitive to the concentration of Cl^- in the medium (Damoder et al., 1986; Ono et al., 1986; Yachandra et al., 1986; Beck & Brudvig, 1988; Haddy et al., 1989; Baumgarten et al., 1990). In Figure 5 the effects of the removal and readdition of Cl^- to PS II membranes were investigated using EPR. To study the effects of removal of Cl^- from PS II, the membranes were washed twice in Cl^--free buffer in order to remove unspecifically associated Cl^-, leaving only the tightly bound Cl^- (washed membranes). As was previously shown in Lindberg et al. (1993) the oxygen-evolution activity in Cl^--free medium was not affected by this treatment compared to the activity in 25 mM Cl^-, provided the washing procedure was quick to avoid loss of the tightly bound Cl^-. Figure 5b shows that the distribution of the EPR signals of the S_2 state after the washing was not significantly altered compared to the control in Figure 5a.

When the washed membranes were dialyzed against a Cl^--free medium, the tightly bound Cl^- slowly dissociated from PS II accompanied by a simultaneous decrease in oxygen-evolution activity to 35% of the control as discussed above. The dialysis procedure induced a change in the relative

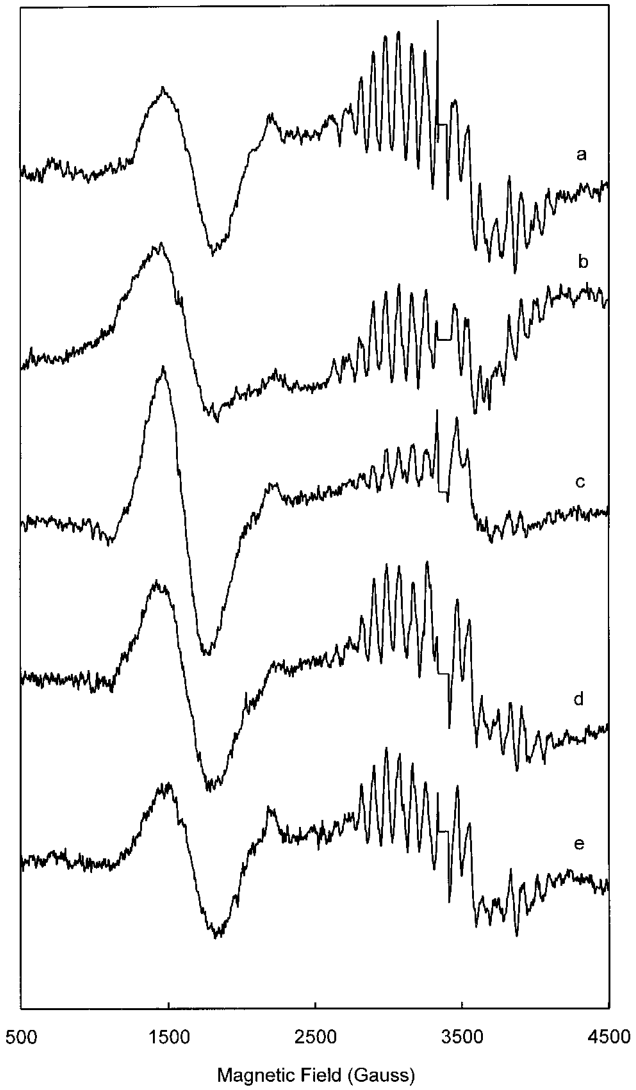

FIGURE 5: Effects on EPR signals of removal and readdition of Cl^- to PS II membranes. (a) PS II membranes washed once and incubated for 3 h in 5 mM NaCl. (b) Membranes from a, washed twice in Cl^--free buffer and frozen within 15 min of the first dilution in Cl^--free buffer. (c) PS II membranes dialyzed for 18 h against Cl^--free buffer. (d) Membranes from c with 5 mM NaCl added. (e) Membranes from (c) with 5 mM NaBr added. Samples in d and e were frozen 30 s after addition of Cl^-- and Br^-. EPR conditions as in Materials and Methods.

intensities of the S_2 state EPR signals from those with saturating concentrations of Cl^- present (Figure 5a), to a shift in intensity toward the $g = 4.1$ signal at the expense of the multiline signal (Figure 5c). When Cl^- was added back to the membranes both the oxygen-evolution activity and the original distribution of the S_2 state EPR signals (Figure 5d) were immediately (<30 s) restored.

Competition with Br^- and F^-

We have previously shown that a few anions, such as Br^-, NO_3^-, and F^-, are able to compete with the binding of the slowly exchanging Cl^- (Lindberg et al., 1993). In Figure 6 we have investigated the labeling of washed PS II membranes with $^{36}Cl^-$ in the presence of Br^-, F^-, or cold Cl^-. As a control for the maximum labeling efficiency, one sample was also incubated with only $^{36}Cl^-$ in the absence of additional anions. The low labeling with $^{36}Cl^-$ in the cold Cl^--medium is due to the dilution of the label which contributed only by 4% to the total amount of Cl^- present in the buffer. As can be seen in Figure 6, Br^- is a good substitute for Cl^- indicated by the decrease in labeling efficiency by about 85%. This is also evident in Figure 5e where Br^- was added to the dialyzed PS II membranes. The relative intensities of the S_2 state EPR signals were immediately returned to those of the control in Figure 5a or in 5d.

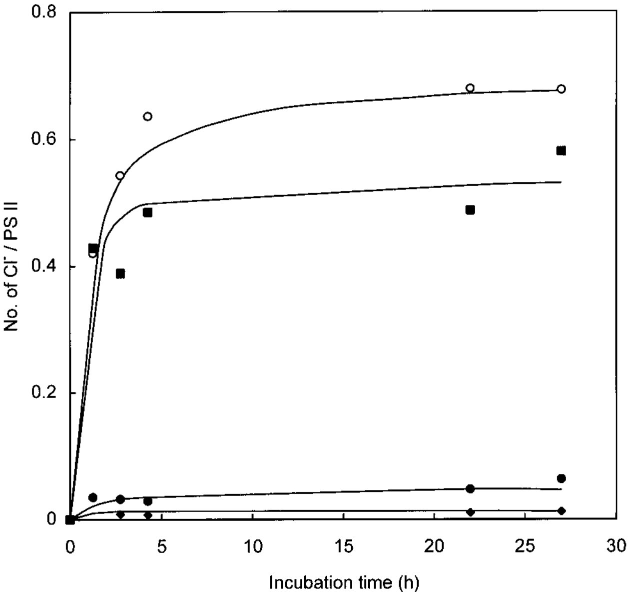

FIGURE 6: Time course for the binding of different anions in the competition with $^{36}Cl^-$ to PS II membranes. PS II membranes were washed by centrifugation three times in Cl^--free buffer and once in buffer with 5 mM of the anion included. The curves show the incorporation of radioactive Cl^- in the washed membranes incubated in (○) Cl^--free buffer; (■) 5 mM NaF; (●) 5 mM NaBr, and (◆) 5 mM NaCl in the presence of 200 μM NaCl (42% $Na^{36}Cl$). Samples were withdrawn at the times indicated and bound $^{36}Cl^-$ was determined as in Lindberg et al. (1990).

However, F^- appears to be a poor competitor for Cl^-, even after prolonged incubation. In order to investigate the competition with Cl^- further, i.e., the ability of F^- to prevent $^{36}Cl^-$ from binding, the binding of $^{36}Cl^-$ at different concentrations of F^- was investigated (Figure 7). The binding of Cl^- was gradually prevented by increasing concentrations of F^-. At 50 mM of F^- the concentration of Cl^- contributed 0.4% of the total halogenide anion concentration but labeled more than 25% of the centers. Our results indicate that F^- competes with Cl^-, but associates with much weaker affinity.

The effects of adding F^- to PS II membranes were also investigated by EPR. The addition of F^- to washed PS II membranes (with the slowly exchanging Cl^- still bound) did not influence the EPR signals much compared to the original intensities (cf. Figures 5b and 8a. The slight decrease in the intensity of the multiline signal compared to the control (Figure 5a) most likely resulted from the loss of a small amount of Cl^- during the sample preparation. In Figures 8b−d, F^- was added to dialyzed, Cl^--free PS II membranes and incubated for 3 h (c, d). In Figure 8b,c, the multiline signal was almost completely eliminated and the $g = 4.1$ signal was increased to 200−300% of the control in Figure 5a. However, all three spectra are very similar to the spectrum from dialyzed, Cl^--free membranes in Figure 5c, which also showed a large increase of the $g = 4.1$ signal at the expense of the multiline. When Cl^- was added to the membranes which had been incubated in the presence of F^-,

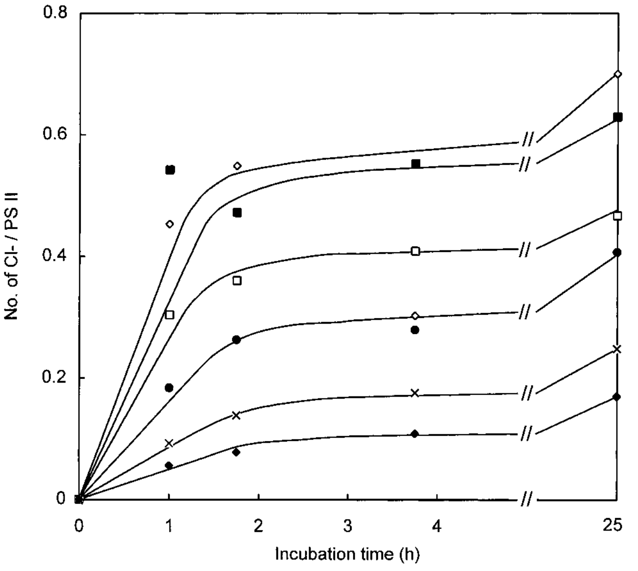

FIGURE 7: Time course for the binding of $^{36}\mathrm{Cl}^-$ in to PS II membranes in the presence of different concentrations of NaF. The PS II membranes were washed by centrifugation three times in Cl^--free buffer and once in buffer with NaF at the below concentrations. The membranes were then incubated in Cl^--free buffer (◇), 1 mM NaF (■), 5 mM NaF (□), 10 mM NaF (●), 25 mM NaF (×) and 50 mM NaF (◆) with 200 μM NaCl (42% $\mathrm{Na}^{36}\mathrm{Cl}$) included. Samples were withdrawn and the amount of incorporated $^{36}\mathrm{Cl}^-$ was determined as in Lindberg et al. (1990).

an immediate decrease in the $g = 4.1$ EPR signal and a concomitant increase in the multiline signal were observed. The redistribution shows that partial replacement with Cl^- occurred and that the dissociation of F^- is rapid under these conditions in contrast to the dissociation of Cl^- (cf. Figure 8a and 8d).

To further investigate the type of binding (i.e., low- or high-affinity) that applies to Br^- and F^-, the time for replacement of Br^- and F^- with Cl^- was measured (Figure 9). Washed PS II membranes were incubated overnight in the presence Br^-, F^-, and Cl^-. One sample was dialyzed against Cl^--free buffer. After incubation the samples were washed with Cl^--free buffer, removing all unspecifically bound anions and leaving only any tightly bound anions. $^{36}\mathrm{Cl}^-$ was added, and the time for incorporation of the label was measured. In this experiment, binding of $^{36}\mathrm{Cl}^-$ to the dialyzed membranes and PS II membranes incubated with Cl^- represent low- and high-affinity binding, respectively (cf. Figure 3). From the results in Figure 9 it can be seen that in F^--treated membranes the binding of $^{36}\mathrm{Cl}^-$ has a $t_{1/2}$ of less than 10 min, similar to the binding to the Cl^--free membranes. F^- has most likely been lost already during washing. This further supports the observations from the experiments in Figures 6, 7, and 8d that F^- binds to PS II with a very weak affinity.

Br^-, however, appears to bind to PS II with high affinity as the $t_{1/2}$ for the binding of $^{36}\mathrm{Cl}^-$ is close to 1 h for the PS II membranes incubated in Br^-, which is similar to the rate observed for replacement of high-affinity Cl^- (Figure 3).

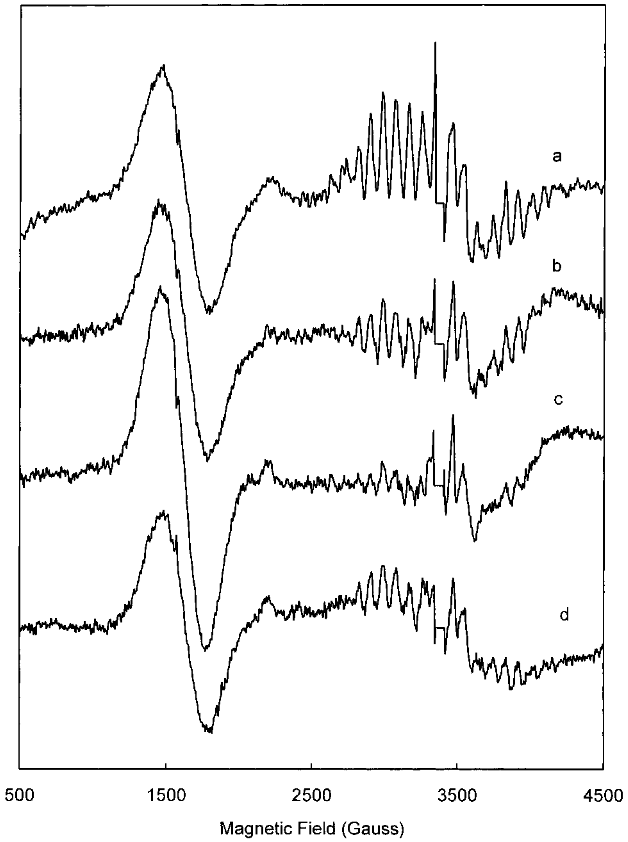

FIGURE 8: Effects of the addition of NaF to PS II membranes. (a) Membranes from Figure 5a were washed twice in Cl^--free buffer with 5 mM NaF. The sample was frozen within 15 min after start of treatment. (b) 5 mM NaF was added to Cl^--free membranes from Figure 5c, and the sample was frozen within 30 s. (c) Cl^--free membranes from 5c were incubated in 5 mM NaF for 3 h. (d) 5 mM of NaCl was added to membranes from c and the sample was frozen within 30 s. EPR conditions as in Figure 5.

DISCUSSION

In summary, from our experiments we draw the conclusion that Cl^--binding responsible for high oxygen-evolution activity in PS II and normal EPR properties of the S_2 state may occur either as high-affinity ($K_\mathrm{d} = 20$ μM) and slowly exchanging ($t_{1/2} = 1$ h) or as low-affinity ($K_\mathrm{d} = 0.5$ mM) and rapidly exchanging (seconds) as illustrated in Figure 10. The fact that Cl^- is able to bind to PS II at different rates and with different binding constants indicates that the reactivation by Cl^- may occur either at two sites with different binding properties where, depending on the conditions, either site is responsible for maintaining the activity, or alternatively, at one site with changing properties. Although our observations do not rule out the two-site model, the simplest explanation for our observations is provided by the one-site, two-state model.

We suggest that the high-affinity state is the normal state of binding. This is the state for Cl^--binding observed when PS II membranes are isolated from spinach grown on medium containing $^{36}\mathrm{Cl}^-$ (Lindberg et al., 1990). The high-affinity state of the site is that labeled in our Cl^--exchange experiments (Figure 3). At the end of the labeling procedure and after the excess of $^{36}\mathrm{Cl}^-$ has been removed, properties such as the intensities of the S_2 state EPR signals (not shown) and high oxygen-evolution activity are the same as those observed in the native material. Not only are the very low concentrations of Cl^- needed to saturate the binding site in this state of PS II able to support maximal activity (Figure 2) but PS II membranes, kept in darkness at 0 °C in the presence of Cl^--concentrations just high enough to saturate the high-affinity site, were kept fully active for several days

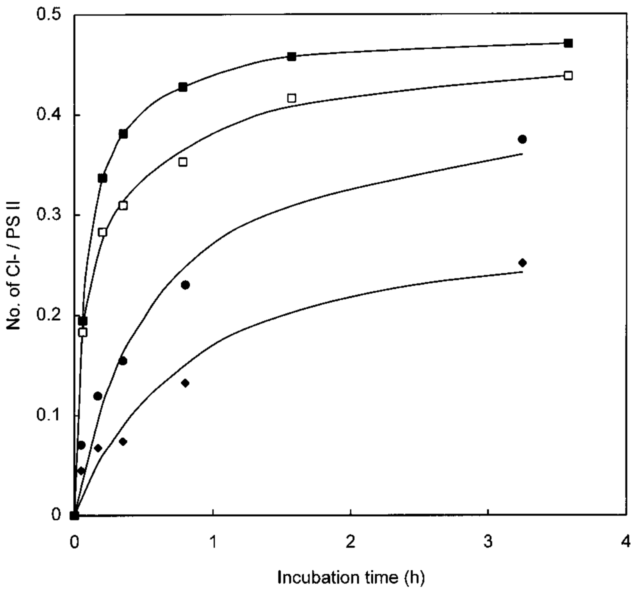

FIGURE 9: Time course for the replacement of Br^- and F^- with Cl^-. Washed PS II membranes were incubated with 5 mM NaBr (◆), 5 mM NaCl (●), 5 mM NaF (■) and in Cl^--free buffer (□) for 14 h. The membranes were washed three times to remove the unbound anions and 200 μM NaCl (42% $Na^{36}Cl$) was added. Samples were withdrawn at the times indicated and the amount of incorporated $^{36}Cl^-$ was determined as in Lindberg et al. (1990).

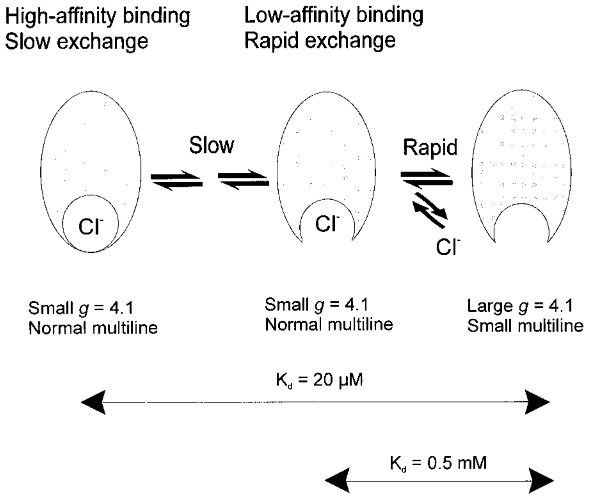

FIGURE 10: Model for the binding of Cl^- to photosystem II. Details of the model are described in the text.

when assayed at the same Cl^- concentrations, whereas Cl^--free membranes showed a strong tendency for activity loss over longer times (data not shown). The lower stability of the water-oxidation complex in the absence of Cl^- agrees with our earlier observations (Lindberg et al., 1993). Chloride has also been observed to protect PS II against heat inactivation (Coleman et al., 1984).

In a recent paper by van Vliet and Rutherford (1996) it is implied that the slowly exchanging 1 Cl^-/PS II is absolutely essential for oxygen-evolving activity. They base their suggestion on the fact that according to Figure 1 in Lindberg et al. (1990) a fraction of centers (30 %) contain Cl^- at pH 6.3 even after 10 h of incubation, and they propose that this residual Cl^- contributes to the 30% oxygen-evolution activity that is observed in Figure 5 (Lindberg et al., 1993). Since the residual Cl^- content in our earlier experiments (Lindberg et al., 1990) was close to the detection limit, we have repeated the $^{36}Cl^-$ incorporation-during-growth experiment, and Table 1 clearly shows that all tightly bound Cl^- in PS II membranes isolated from $^{36}Cl^-$-labeled spinach is exchanged with surrounding Cl^- in 18 h. Furthermore, Figure 1 in this work clearly supports our view that Cl^- is not an absolute requirement for oxygen-evolution. PS II membranes that have been depleted of Cl^- by the dialysis method are partially active in the absence of Cl^- as nearly 100% of the centers are able to evolve oxygen although at a reduced rate, as was found in Lindberg et al., (1993). The kinetic behavior of the Cl^--depleted membranes is not that expected for a membrane preparation composed of a mixture of fully active (Cl^--containing) and inactive (Cl^--free) centers (see dashed line in Figure 1) . In addition, the O_2-evolution activity is not permanently lowered by the dialysis method but can be restored by adding back Cl^- at low concentrations. This shows that the Cl^--binding site has not been permanently altered, and therefore the slowly exchanging, high-affinity Cl^- is able to re-bind with time to normal levels (Figure 3). The dialysis method for removal of Cl^- should be compared to other Cl^--depletion methods which are commonly employed, such as high pH in combination with sulfate (Ono et al., 1986) or washing with high concentrations of salt (Kuwabara & Murata, 1983; Ono & Inoue, 1983; Homann, 1988b). These treatments are likely to irreversibly affect PS II, as the activity can only be sustained in the presence of high concentrations of Cl^-. The dissociation constant for Cl^- after such treatments is generally in the order of 1 mM or higher (Homann, 1985; Miyao & Murata, 1985; Homann, 1988a). The situation after using these depletion methods is in some respects similar to that after removal of Cl^- by dialysis in that, initially, high concentrations of Cl^- are needed for water-oxidation and the response on addition of Cl^- is immediate. An important difference is that in the high-pH, sulfate-treated membranes the ability to bind Cl^- in the slowly exchanging, high-affinity mode is permanently lost, probably due to the partial loss or other perturbation of the three extrinsic subunits (Lindberg et al., 1993), whereas the dialyzed membranes in the presence of Cl^- slowly return to their initial tight-binding state. When the Cl^--binding site has been emptied and converted to the low-affinity state, one can view this as a transformation of the site into a more open conformation with easy access to the anion binding site as the addition of Cl^- is able to restore the original S_2 state EPR signals (Figure 5d) and high water-oxidizing activity (Figure 2) within seconds.

The picture of an open conformation with an easily accessible anion-binding site is also consistent with the initial, prompt dissociation of the activating Cl^- from the low-affinity site following the transfer of the membranes from medium containing Cl^- to the Cl^--free environment (Figure 4).

With prolonged incubation in the presence of Cl^-, a larger fraction of the available high-affinity, slowly exchanging sites becomes occupied (Figure 3). Consequently an increasing fraction of the activity remains when the membranes are diluted in Cl^--free medium as the Cl^- ion cannot easily escape any longer (Figure 4). The rate of transition from the low- to high-affinity state seems to be dependent on the concentration of Cl^- in the medium (Figure 4), increasing with increasing Cl^- concentrations. In other words, Cl^- bound in the low-affinity state appears to be a prerequisite for transformation into the high-affinity state in which the Cl^- ion is locked into a closed conformation.

The S_2 state EPR signals were not significantly altered in washed membranes (Figure 5b) compared to the control (Figure 5a). Contradictory results were found by van Vliet and Rutherford (1996) in that they sometimes observed a large increase of the $g = 4.1$ EPR signal in addition to a significant lowering of the S_2 multiline signal after Cl^--free washes while occasionally this was not seen. The various results concerning the amplitude of the $g = 4.1$ signal obtained by van Vliet and Rutherford after washing with Cl^--free buffer is easily explained (cf. Figures 5b and 5c in this work). A short exposure to Cl^--free conditions will not lead to any substantial loss of Cl^- and, consequently, will have little effect on the distribution of the S_2 state signals. More extended washing will result in dissociation of Cl^- from its binding site and an increase in the $g = 4.1$ signal amplitude. This further emphasizes the need for keeping track of the time used for washing. In our experiments we always try to keep the washing procedure as short as possible to avoid loss of Cl^-. Occasionally an increased number of samples handled simultaneously will require a longer time for washing and consequently lead to an apparent lower labeling of PS II (cf. Figures 3 and 9). The amount of time needed for washing also explains why in our experiments we always find less than one Cl^-/PS II. From the known kinetics for release of bound Cl^- the maximal amount of Cl^- bound at the time of dilution in Cl^--free medium can be extrapolated to 0.8−1 Cl^-/PS II. Also, one can expect that a small fraction of centers is damaged or inactive and consequently not able to bind Cl^-.

A shift in the equilibrium between the two forms in EPR exhibiting the S_2 state, $g = 4.1$, and multiline, toward $g = 4.1$ (Figure 5c) after complete removal of Cl^- from PS II membranes has also been noted earlier as a result of the use of high pH and sulfate to remove Cl^- (Ono et al., 1986; Beck & Brudvig, 1988). As was noted above, this Cl^--depletion method may result in the loss of significant amounts of the 16, 24, and 33 kDa proteins in addition to permanently eliminating the ability of the binding site to convert to the high-affinity, slowly exchanging state (Lindberg et al., 1993). Differences between our results and some other observations, e.g., the absence of effects on the intensity of the $g = 4.1$ EPR signal after Cl^--depletion (Ono et al., 1986), may therefore be traced to differences imposed on the photosynthetic material by the various depletion methods.

From the EPR spectra alone it is not possible to distinguish between low- and high-affinity Cl^- binding. In Figure 5b only the high-affinity, slowly exchanging Cl^- is present, and in Figure 5d only the low-affinity, rapidly exchanging Cl^- is present. In both cases the equilibrium is shifted toward the multiline conformation, and the shapes of the EPR spectra are indistinguishable. This was also noted by Ono et al. (1986), after the readdition of Cl^- to high-pH/sulfate-treated PS II membranes.

Bromide seems to be as efficient as Cl^- in maintaining the original distribution of the S_2 state EPR signals (Figure 5e). Similar results were obtained by Yachandra et al. (1986), Beck and Brudvig (1988), and Haddy et al. (1989). F^-, however, as can be seen in Figure 8b−d, shifts the equilibrium toward the $g = 4.1$ signal (Casey & Sauer, 1984; Damoder et al., 1986; Yachandra et al., 1986; Baumgarten et al., 1990; DeRose et al., 1995). Figures 6 and 9 indicate that Br^- is incorporated in PS II membranes as efficiently as Cl^-, whereas F^-, although it competes with Cl^-, binds with much lower affinity. Chloride is able to restore the S_2 state EPR signals immediately after the addition to membranes incubated in F^- (cf. Figures 8c, 8d, and 5a). The lower than maximal reconstitution of the original balance between the EPR signals further emphasizes the competition between the anions in the open low-affinity state. The addition of F^- to washed PS II membranes, i.e., membranes with the high-affinity, slowly exchanging Cl^- still bound, causes virtually no effects on the signals (cf. Figures 8a and 5a). The different effects caused by Cl^- and Br^- on the one hand and F^- on the other hand after the addition to PS II membranes suggest different modes of binding for the anions. The immediate displacement of F^- by Cl^- in membranes treated for several hours with F^- (Figure 9) indicates that F^- binds with weaker affinity in a more open conformation than Cl^- and that by binding to PS II F^- stabilizes the low-affinity, rapidly exchanging binding site for anions. The somewhat faster labeling of F^--treated membranes compared to dialyzed membranes (Figure 9) may appear surprising. However, as may be seen from the EPR spectra (cf. Figures 5c and 8c), treatment with F^- appears to emphasize the effects accomplished by dialysis, i.e., a further shift in EPR signal intensities. It cannot be ruled out that the F^--induced EPR spectrum reflects F^--dependent structural changes which facilitate anion exchange and the binding of Cl^-.

The slow replacement of Br^- by Cl^- (Figure 9) points to a high-affinity, closed mode of binding for Br^- similar to that observed for Cl^-.

The two states of anion-binding which we report here should not be confused with the two conformations associated with the binding of halides, ammonia, and amines which has been discussed by Beck and Brudvig (1988). Due to their use of high-pH/sulfate treatment to remove Cl^-, the reported ligand exchange concerns only an open low-affinity conformation that is similar to but not identical to ours.

In conclusion, of the two modes of Cl^--binding, responsible for high oxygen-evolution activity and normal EPR properties of the S_2 state, the high-affinity ($K_d = 20\ \mu M$), slowly exchanging ($t_{1/2} = 1$ h) is the normal state of binding, but once Cl^- has been removed, it will first rebind as low-affinity ($K_d = 0.5$ mM), rapidly exchanging ($t_{1/2} < 15$ s) followed by conversion into a high-affinity, slowly exchanging mode of binding.

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