S-State Dependence of Chloride Binding Affinities and Exchange Dynamics in the Intact and Polypeptide-Depleted O_2 Evolving Complex of Photosystem II†

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ABSTRACT: The Cl^- binding properties in the successive oxidation states of the O_2 evolving complex of photosystem II were investigated by measurements of UV absorbance changes, induced by a series of saturating flashes, that monitor manganese oxidation state transitions. In dark-adapted, intact photosystem II, Cl^- can be replaced by NO_3^- in minutes, in an exchange reaction that depends on the NO_3^- concentration and that is not rate-limited by dissociation of Cl^- from its binding site. Preillumination of dark-adapted photosystem II by one or two flashes accelerated the NO_3^- substitution reaction by an order of magnitude. A quantitative analysis of the Cl^- concentration dependence of UV absorbance changes, measured in photosystem II preparations depleted of extrinsic 17 and 23 kDa polypeptides, shows that the Cl^- binding properties of photosystem II change with the oxidation state of the oxygen evolving complex. Although the affinity for the individual S-states could not be determined with precision, it is shown that the affinity is an order of magnitude lower in the S_2 state than in the S_1 state. Comparison of the results obtained using intact photosystem II and preparations depleted of the 17 and 23 kDa extrinsic polypeptides suggests that these proteins constitute a diffusion barrier, which prevents fast equilibration of the Cl^- binding site with the medium, but does not change the Cl^- affinity of the binding site.

Photosystem II (PSII)[1] is a multisubunit, membrane-bound enzyme system that couples light absorption to the oxidation of H_2O and the reduction of plastoquinone. The O_2 evolving reaction involves a number of components that are collectively called the O_2 evolving complex (OEC).[1] Suspended in a framework comprised of both intrinsic and extrinsic polypeptides, the active site of the complex is associated with a cluster of four Mn atoms along with one atom each of Ca^{2+} and of Cl^- (*1*−*3*). The structure of the complex is unknown, but EPR and X-ray absorption studies (*4*) have led to detailed proposals for the structure of the Mn cluster and its ligand environment (*5*). The Mn is involved in the stepwise accumulation of oxidizing equivalents, which raise the oxidation state of the complex in terms of the Kok model (*6*) from S_0 to S_3. In darkness, the system relaxes to S_1, in which the Mn valency is proposed to be III, III, IV, IV (*5*, *7*). The $S_1 \rightarrow S_2$ transition has been identified as a Mn^{III} to Mn^{IV} oxidation state advancement (reviewed in *4*). $S_0 \rightarrow S_1$ may involve Mn^{II} to Mn^{III} and $S_2 \rightarrow S_3$ Mn^{III} to Mn^{IV}, but these assignments are more controversial, although an absorption increase in the UV region as well as a shift to higher energy of the Mn K-edge in X-ray absorption strongly implicates Mn involvement in these S-state transitions (see *8* for a detailed discussion). Light-induced charge separation by PSII in the S_3 state, as in the lower oxidation states, causes a transient oxidation of Y_Z (*1*), the tyrosine residue that acts as the secondary electron donor in PSII. In the S_3 state, however, this is followed in a few milliseconds by simultaneous reduction, by H_2O, both of the tyrosine and of S_3 to S_0.

Calcium and chloride are essential cofactors for O_2 evolution. Although the Cl^- requirement for photosynthetic O_2 evolution has been studied intensively (*8*−*11*), the binding site for this cofactor has not yet been identified with certainty and its role in H_2O oxidation is unknown. Several observations suggest that Cl^- binds in the vicinity of the Mn cluster. Removal of the halide results in a change in the magnetic properties of the Mn complex. Chloride-depleted PSII reaction centers show no EPR multiline signal (*12*), although it can be shown by optical and X-ray absorption spectroscopies that oxidation of Mn has taken place (*13*, *14*, but see *15*, *16*). The observation that Cl^-, primary amines, and other Lewis bases compete for the same binding site was taken as an indication that Cl^- binds directly to a metal (*17*). The effectiveness of amines in the competition was proportional to their basicity, suggesting that they bind as Lewis bases to a Lewis acid, in this case Mn in an oxidation state higher than 2+. Certain EXAFS results may support this conclusion. The group of Klein reported (*18*,*19*) that the differences in EXAFS spectra of cyanobacteria grown on Br^- or Cl^-

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen evolving complex; PSII, photosystem II; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Y_Z, the tyrosine residue that acts as the primary electron donor to P_{680}.

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were consistent with one Mn-associated halide ligand. Similarly, small Mn EXAFS changes upon inhibition by F^- were interpreted as being consistent with F^- binding to Mn (*20*). However, these changes upon halide substitution are too subtle to provide strong evidence concerning the identity of the Cl^- binding site. Recently, Force et al. (*21*) determined by pulsed EPR measurements that acetate bound to the OEC is in close (ca. 3.5 Å) proximity to $Y_Z^{\bullet}$. Since acetate displaces Cl^- by a competitive mechanism, these authors concluded that Cl^- must be located in close proximity to both Y_Z and the PSII Mn cluster. If Cl^- is ligated to Mn, one would expect the Cl^- binding affinity to depend on the oxidation state of the metal, although it is not immediately obvious in which direction; the affinity might increase due to larger ligand field stabilization energy associated with higher oxidation states of manganese or, alternatively, decrease due to increasing hardness of high-valent Mn (*22*).

Unfortunately most of the methods utilized to investigate the role of Cl^- in activating the OEC do not provide a means by which to carry out measurements as a function of S-state, mainly because of the short lifetimes of the S_2 and S_3 states. There is only one report in which Cl^- affinity has been monitored as a function of S-state, the $^{35}Cl^-$ NMR study by Preston and Pace (*23*). Photosystem II preparations which did not retain the extrinsic 17 and 23 kDa polypeptides were found to bind Cl^- in the S_2 and S_3 states with an affinity comparable to that observed for activation of O_2 evolution, while no such binding was found in the lower S-states. Also, the lower S-states do not appear to require Cl^- for activity: using PSII membranes from which the 17 and 23 extrinsic polypeptides were dissociated by high pH/sulfate treatment, we have shown by UV absorbance difference spectroscopy that both the oxidation of S_2 to S_3 and that of S_3 to $S_0 + O_2$ require the presence of Cl^-, while the oxidation of S_0 to S_1 and that of S_1 to S_2 do not (*14*). However, this result does not exclude the possibility that Cl^- is in fact bound in the lower S states. The $^{35}Cl^-$ NMR result could also be explained by a high Cl^- affinity accompanied by slow exchange of the halide in the lower S-states, because exchange slower than a few milliseconds would be undetectable by NMR. Such an interpretation would reconcile the NMR data with the findings of Lindberg et al. (*3*), who determined a K_d (dissociation constant) of 20 μM and a dissociation time of 1 h for Cl^-, using intact, dark-adapted (low S-state) PSII membranes. On the other hand, this result is highly dependent on the integrity of photosystem II. Removal of the extrinsic 17 and 23 kDa polypeptides abolishes the high-affinity, slowly exchanging Cl^- binding behavior of S_1 (*3*). Results from earlier studies are consistent with this finding. Polypeptide-depleted PSII membranes require high concentrations of added Cl^- for maximum activity (*24, 25*), and the Cl^- binding site appears to be rapidly accessible to anions, consistent with the finding that polypeptide depletion of the OEC renders it more accessible in S_1 to reducing agents that attack Mn and destroy activity (*26*).

If Cl^- exchange in the S_1 state is limited by the 1 h release time reported by Lindberg et al. (*3, 27*), then other data obtained with intact PSII preparations must be taken to suggest that the rate of Cl^- exchange with the OEC depends on turnovers of the S-states. Many O_2 evolution studies of the competition between Cl^- and substituting anions (*17, 28*−*30*) or amines (*31*) suggest that in one or more S-states the halide must be susceptible to rapid exchange in the presence of extrinsic polypeptides. A further complication is that release of Cl^- itself, without removal of the polypeptides, has been reported to change the properties of the binding site to lower affinity (0.5 mM) and fast exchange (<15 s) (*27*).

To further explore and clarify some of these unresolved issues that relate to Cl^- affinity and exchange dynamics in PSII, we have carried out measurements of the kinetics of UV absorbance changes induced in intact and polypeptide-depleted PSII membranes by illumination with a series of saturating flashes. In intact PSII, the substitution of NO_3^- for Cl^- was studied making use of the observation by Sinclair (*32*), confirmed and extended here, that NO_3^- substitution specifically retards the $S_3 \rightarrow S_0$ transition. In polypeptide-depleted PSII, the Cl^- concentration dependence of the kinetics of the S-state cycle was investigated. The results indicate that Cl^- exchange at the binding site itself takes place in seconds, and appears to be much slower in intact PSII because the extrinsic proteins constitute a diffusion barrier shielding the site from the external medium. The Cl^- affinity is shown to depend on S-state and pH, but not on the presence of extrinsic proteins.

MATERIALS AND METHODS

Photosystem II membrane fragments were prepared from spinach as described by Berthold et al. (*33*) with modifications (*34*), and stored at 77 K in buffer containing 20 mM MES, pH 6.0, and 0.4 M sucrose at 3−4 mg of Chl/mL. Cl^-/NO_3^- exchange was probed at pH 6.0 using these intact PSII membrane fragments, diluted to 0.2 mg of Chl/mL in 0.4 mM sucrose and 25 mM MES. Dissociation of Cl^- and the 17 and 23 kDa extrinsic polypeptides was achieved by incubating intact PSII membranes for 15 min in a buffer containing 50 mM Hepes, pH 7.5, 50 mM Na_2SO_4, and 0.3 M sucrose. The incubation was carried out in the dark at 0 °C at a Chl concentration of 0.2 mg/mL. These samples were probed by UV spectroscopy immediately after incubation. For measurements at pH 6.0 (Table 1), incubation was followed by centrifugation; the pellet was resuspended at pH 6.0 in a buffer containing 25 mM MES and 0.4 M sucrose. For all types of measurements, Cl^- or NO_3^- were added as Na salts.

Flash-induced absorbance difference measurements were carried out at room temperature in a single-beam apparatus described previously (*35*). The optical path length was 1.2 mm. Saturating excitation flashes were generated by a Nd:YAG laser (532 nm, half-width 6 ns); flash repetition rates are given in the figure legends. The S-state advances were probed at 295 nm, where absorbance changes due to the acceptor side of PSII are minimal. The artificial electron acceptors DCBQ and ferricyanide (final concentrations: 50 and 100 μM, respectively) were added to avoid limitations from the PSII acceptor side. Steady-state rates of O_2 evolution were assayed with a Clark electrode at 20 °C, in the presence of 2.5 mM ferricyanide and 0.4 mM DCBQ at a Chl concentration of 20 μg/mL. For these measurements, the Cl^--depletion procedure was shortened to 1 min; this time was sufficient to completely block O_2 evolution. The residual Cl^- contamination of buffers was determined as described by Lindberg et al. (*3*) and was routinely found to be in a range of 20−40 μM.

For the computer simulations of S-state turnover according to the Kok model (*6*), parameter values were optimized to fit the measured absorbance changes by minimizing the mean square difference between fit and data, using the NAG Library routine E04JAF. Data used for the fitting procedure consisted of 'stable' absorbance changes, measured 40 ms after the flash, but some data on the millisecond transient reflecting $S_3 \rightarrow S_0$ were included to fix the phase of the S-state cycle. If this is not done, the phase cannot be determined since no assumptions are made about the initial S-state distribution or the relative extinction coefficients of the successive S-state transitions. On the basis of our previous study (*14*), it was assumed in the simulations that a charge separation in a Cl^--deficient PSII reaction center in S_2 or S_3 does not advance the S-state but oxidizes Y_Z and decays by charge recombination (to 65% at 40 ms after the flash, as determined from the measurements without Cl^-). The probability that Cl^- is bound to its site at the moment of the flash was calculated explicitly from the Cl^- concentration in the medium using the on (second order) and off (first order) rate constants k_{on} and k_{off} obtained from the equilibrium constant of dissociation K_d ($=k_{off}/k_{on}$), and the time constant of dissociation τ_d ($=1/k_{off}$). K_d and τ_d were fit parameters. Additional fit parameters used were the probability of misses (S-state- and Cl^--independent) and of S-state advance between the flashes due to the measuring light. Double hits were not allowed, on account of the short flashes used. The lifetimes of the S_2 ($\pm Cl^-$) and S_3 states were determined in separate experiments. Data from the first flash were disregarded in the fitting routine to avoid complications from nonoscillating absorbance changes due to inactive centers (*36*). All measurements simulated simultaneously in Figure 8 differ only in preillumination and Cl^- addition, not in the value of any adjustable parameter.

RESULTS

Cl^- Exchange Dynamics in Intact PSII. Using the method of Bouges-Bocquet (*37*) to determine the rate-limiting step for each S-state transition from O_2 yields under flash illumination, Sinclair (*32*) showed that NO_3^- substitution for Cl^- in thylakoids specifically retarded the $S_3 \rightarrow S_0$ transition. The kinetics of this transition can be directly monitored by the absorbance at 295 nm on illumination of dark-adapted PSII by three saturating flashes. Stable absorption increases due to the oxidation of the complex up to the S_3 state appear on the first two flashes, whereas the third flash, after an initial increase due to Y_Z oxidation (as well as $S_2 \rightarrow S_3$ transitions in those centers that were still in S_2 after the second flash), is followed by simultaneous reduction of $Y_Z^{\bullet}$ and the Mn cluster. This causes a large absorbance decrease with a time constant of a few milliseconds and an amplitude proportional to the O_2 yield (*38*). Figure 1 shows that the absorbance decrease on the third flash was slowed by a factor of 3 when NO_3^- replaced Cl^- in both intact (left traces, measured after 1 h incubation with the anion) and polypeptide/Cl^--depleted PSII membrane fragments (right traces, measured after addition of 50 mM Cl^- or NO_3^-). In the latter preparation, no such absorbance decrease was seen when neither Cl^- nor NO_3^- was added (*14*), demonstrating that the slower NO_3^- kinetics are due to PSII centers in which the anion has replaced Cl^-. The slower pH 7.5 kinetics and their somewhat biphasic appearance, as compared to pH 6.0, are also seen in intact PSII (without sulfate treatment), so they are due only to the increased pH, not to removal of Cl^- and polypeptide depletion (not shown).

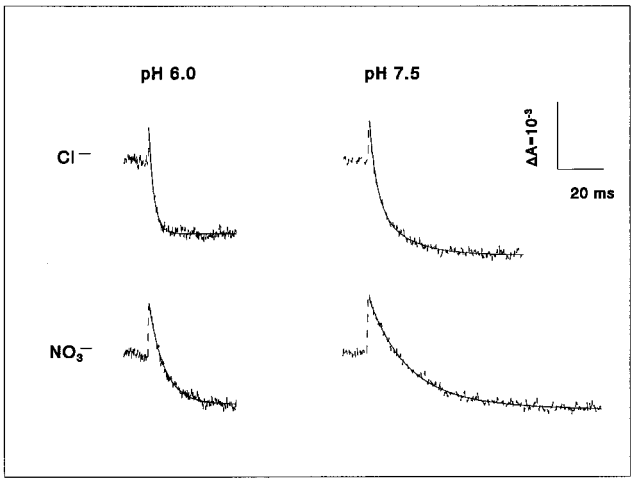

FIGURE 1: Effects of Cl^- or NO_3^- on absorbance changes at 295 nm induced by the third saturating flash in dark-adapted PSII membrane fragments. Left traces: intact PSII at pH 6.0, incubated for 1 h in the presence of 30 mM Cl^- (upper trace) or 30 mM NO_3^- (lower trace). Right traces: PSII Cl^- depleted by pH 7.5/50 mM SO_4^{2-} treatment and reactivated by addition of 50 mM Cl^- (upper trace) or 50 mM NO_3^- (lower trace). The spacing between flashes was 1 s.

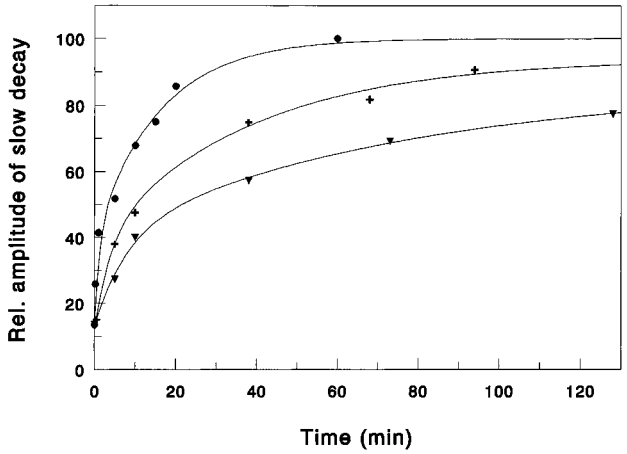

FIGURE 2: Kinetics of Cl^- replacement by NO_3^- in dark-adapted, intact PSII membrane fragments at pH 6.0. The NO_3^- concentrations shown are 5 (triangles), 10 (crosses), and 30 mM (circles), respectively. The relative amplitudes of the slow component in the biexponential fit of the $S_3 \rightarrow S_0$ kinetics, measured as in Figure 1, with fixed time constants of 2.15 ms (Cl^-) and 6.80 ms (NO_3^-) are plotted. The fits shown are biexponential with both rate constants proportional to $[NO_3^-]$, corresponding to second-order rate constants of 4.2×10^{-1} M^{-1} s^{-1} and 4.4×10^{-2} M^{-1} s^{-1}. The spacing between flashes was 100 ms.

The NO_3^- effect on the $S_3 \rightarrow S_0$ kinetics was exploited to determine the extent of substitution of NO_3^- for Cl^- in intact PSII membranes at pH 6.0. This was accomplished using a biexponential fit of the kinetics of the absorbance decrease after the third flash, keeping the two time constants fixed at the values observed with Cl^- (2.15 ms) and after complete substitution by NO_3^- (6.80 ms), respectively. Figure 2 shows the extent of substitution as a function of incubation time after addition of 5, 10, or 30 mM NO_3^- to intact, dark-adapted PSII in Cl^--free medium. In about 14% of reaction centers, the exchange was very fast at all NO_3^- concentra-

tions shown, and was not resolved in our measurements. Since this percentage is independent of NO_3^- concentration, it is possible that these PSII centers are in a different, "open" conformation and do not bind Cl^- tightly. Such change in the properties of the Cl^- site after prolonged incubation in low Cl^- buffers was demonstrated by Lindberg and Andréasson (*27*). In the remaining fraction of centers, Cl^-/NO_3^- exchange proceeds with at least biphasic kinetics which appear to be proportional to the NO_3^- concentration: the times needed for half-exchange range from more than 30 min in the presence of 5 mM NO_3^- down to 8 min at 30 mM. These times are shorter than the 1 h Cl^- release time reported by Lindberg et al. (*3*) for dark-adapted PSII, in Cl^--free media. It should also be noted that, although the NO_3^- concentrations required to obtain these exchange rates are orders of magnitude larger than the reported K_d for Cl^-, the NO_3^- K_d is quite similar to that of Cl^-. After equilibration of intact PSII with a mixture of 30 mM each of Cl^- and NO_3^-, the $S_3 \rightarrow S_0$ kinetics showed about 40% slow phase (data not shown).

A dependence on S-state of the Cl^-/NO_3^- exchange was investigated as follows: 10 mM NO_3^- was added to dark-adapted, intact PSII, and within 10 s after addition, which allows exchange only in 14% of 'open' PSII reaction centers, zero, one, or two flashes were fired and the sequence of three flashes was completed 30 s later. The absorbance decrease induced by the third flash was analyzed as above by fitting of a biexponential decay to determine the extent to which Cl^- had been replaced by NO_3^- (Figure 3). Since only the $S_3 \rightarrow S_0$ transition on the third flash was measured, all centers contributing to the absorption changes in Figure 3A were in S_1 during incubation, in S_2 in panel B, and in S_3 in panel C. These data show that incubation of S_1 in NO_3^- for 30 s led to Cl^- replacement only in the 14% of 'open' PSII reaction centers, and incubation for the same period of time in S_2 and S_3 increased the extent of Cl^- substitution to about 45%. Since the data of Figure 2 show that 10 min in S_1 is required to obtain a 45% displacement of Cl^- by 10 mM NO_3^-, the data of Figure 3B,C also show that Cl^-/NO_3^- exchange is accelerated by an order of magnitude in S_2 and S_3, as compared to S_1.

From the proportionality between substitution rate and NO_3^- concentration in Figure 2, it can be concluded that the intrinsic rate constant of Cl^- release from its binding site in dark-adapted, intact PSII does not limit the rate of exchange of NO_3^-. Therefore, the release time constant must be much shorter than Lindberg et al. (*3*) proposed, if the presence of NO_3^- in the medium does not modify the anion binding site while Cl^- is still bound to it. Since Cl^- is retained by the OEC for a long time in Cl^--free media (*3*), it appears that the occupant of the Cl^- binding site must be in rapid equilibrium with only a small sequestered domain that is maintained in isolation from the bulk medium by a substantial diffusion barrier. It seems logical to assume that the extrinsic 17 and 23 kDa polypeptides either are critical to maintenance of this barrier or are, in fact, the barrier itself.

Characterization of Cl^- Affinities and Exchange Rates in PSII Depleted of Extrinsic Polypeptides. The S-state dependence of the Cl^-/NO_3^- exchange dynamics revealed by the preceding data could in theory arise from conformational changes affecting the integrity of the diffusion barrier between the sequestered domain and the external medium,

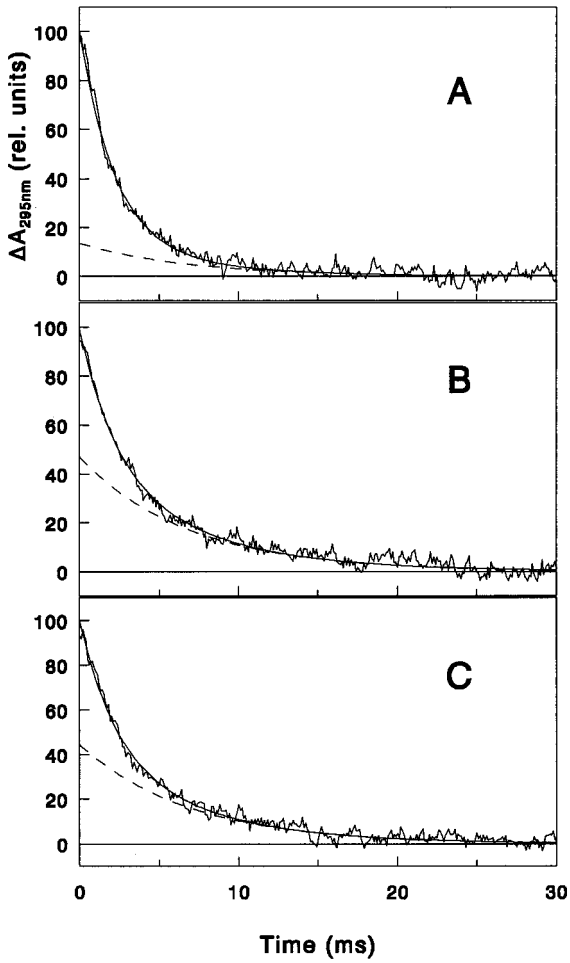

FIGURE 3: Effect of NO_3^- on absorbance changes due to the $S_3 \rightarrow S_0$ transition in dark-adapted intact PSII at pH 6.0. Conditions as in Figure 1 (except that flash frequency was 10 Hz), measured after 30 s incubation with 10 mM NO_3^- (A). In panel B, the sample was given one preflash, and in panel C two preflashes were fired before the incubation [but following the mixing step (10 s)]. Smooth lines are biexponential fits with the fixed time constants used in the determination of the points of Figure 2, indicating 14% slow phase in panel A and 45% slow phase in both panels B and C. Dashed lines indicate the contribution of the slower phase to the total signal amplitude.

or from S-state-dependent properties of the Cl^- binding site itself. The latter possibility was investigated using PSII preparations depleted of the extrinsic 17 and 23 kDa polypeptides by incubation at pH 7.5 in the presence of 50 mM sulfate (as in *14*). Samples prepared this way require millimolar Cl^- for full recovery of O_2 evolution activity, as do other polypeptide-depleted preparations (*24*, *39*), but do not require Ca^{2+} addition for activity, and exhibit a much more pronounced UV absorbance oscillation (*14*) than do salt-washed PSII preparations (*40*).

Determinations of Cl^- affinities in polypeptide-depleted PSII membranes were carried out by measurements of steady-state rates of O_2 evolution and of the amplitude of the oscillation of the 295 nm absorbance changes (Figure 4). In both sets of experiments, 50 mM Cl^- was found to be saturating, but half-maximal activation was reached at about 6.5 mM Cl^- in assays of steady-state activity, and at about 1.5 mM Cl^- in the measurements of flash-induced S-state turnover. The curves in Figure 4 were calculated assuming that activity is proportional to the fraction of centers with

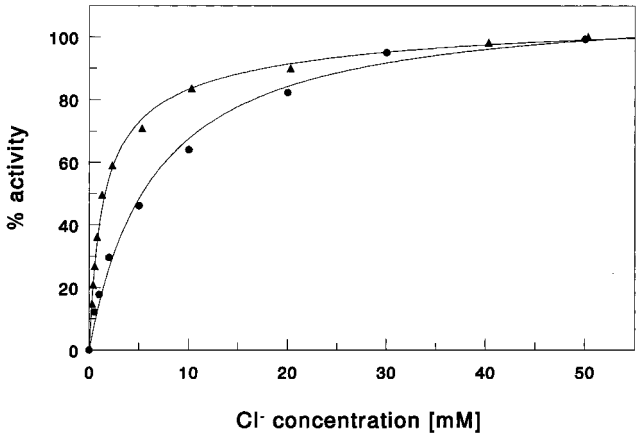

FIGURE 4: Cl^- concentration dependence of O_2 evolution by PSII membrane fragments depleted of extrinsic polypeptides. Circles: rate of O_2 production in saturating light. Triangles: amplitude (reciprocal scaling factor, see Figure 6) of 295 nm absorbance oscillation produced by illumination with a series of saturating flashes at 1 Hz. The 100% rate of O_2 evolution was 280 μmol h^{-1} (mg of Chl)$^{-1}$.

an occupied Cl^- site according to a simple binding equilibrium: $[Cl^-]/([Cl^-] + K_d)$. A dissociation constant (K_d) of 6.5 mM was found for the O_2 evolution rate. For the 295 nm absorbance oscillation amplitude in a flash series, however, heterogeneity of K_d had to be assumed, with values of 1.2 mM and about 20 mM. This finding indicates that the situation is more complicated than measurements under continuous illumination suggest.

In polypeptide-depleted PSII preparations, Cl^- binding took place within the 10 s needed to add and mix the Cl^- in the sample and start the measurement. Addition of nonsaturating Cl^- (1 mM) 10 s or 10 min before measurements of flash-induced oscillations produced no obvious difference in the 295 nm absorbance kinetics (Figure 5A,B, respectively). Therefore, Cl^- exchange in dark-adapted, polypeptide-depleted PSII appears to reach equilibrium in 10 s or less, consistent with the ^{36}Cl result showing that removal of extrinsic PSII polypeptides abolishes slow halide exchange (*3*). If Cl^- exchange at its binding site is sufficiently rapid, then in principle a nonsaturating Cl^- concentration in the medium should randomly decrease the probability that charge separation will cause an S-state transition from S_2 to S_3 or from S_3 to S_0, because those transitions require the presence of Cl^- (*14*). As a consequence, the resulting 'misses' in S_2 and S_3 would damp the oscillation in UV absorbance changes, and lengthen its period.

It appears, however, that although the amplitude of UV absorbance changes is sensitive to the Cl^- concentration, neither the damping nor the period of oscillation of the signals depends on the Cl^- concentration. This is illustrated for two Cl^- concentrations in Figure 6, where the two measurements have been made to overlap and coincide by scaling the amplitude and offset. Although the oscillation amplitude at 0.5 mM Cl^- is only 40% of that observed with saturating Cl^-, active centers oscillate as if they were saturated with Cl^-, while the remaining 60% do not, and instead produce an offset due to Y_Z oxidation on each flash except the first (*14*). This result would suggest that little, if any, exchange of Cl^- ions from Cl^--sufficient to Cl^--deficient reaction centers took place on the time scale of the measurement (10 s), even though the data of Figure 5 indicate

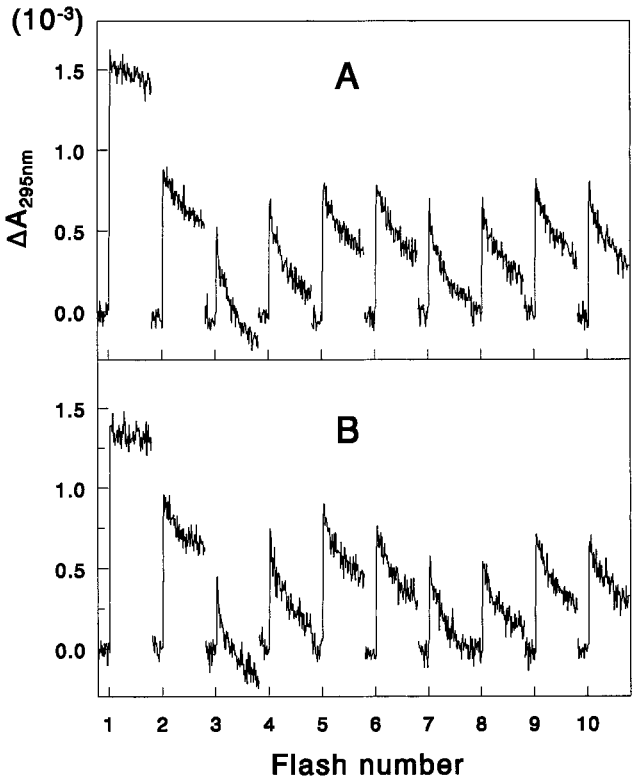

FIGURE 5: Absorbance changes at 295 nm induced by 10 saturating flashes at 1 Hz, starting 10 s (A) or 10 min (B) after addition of 1 mM Cl^-. Each trace runs from 10 ms before the flash to 40 ms after.

that this time should be sufficient for complete exchange at the Cl^- binding site.

It is still possible that Cl^- binding is in fact in rapid equilibrium throughout the measurement, provided that the Cl^--sufficient reaction centers have a much higher affinity for the halide than do Cl^--deficient centers. Since this would have to be true at all Cl^- concentrations tested, a wide distribution of Cl^- affinities in the sample would have to exist. This possibility was examined in detail by computer simulations of S-state turnovers to obtain a simultaneous fit of the measurements in Figure 4 (triangles). The best fit required a set of five K_d values ranging from 0.03 to 10 mM, which is probably not meaningful; in fact, the remaining differences between the fit and the data strongly indicate that such a model is unsatisfactory, as shown in Figure 7A. At both high and low Cl^- concentrations, the residuals are clearly not random, but instead show a period 4 oscillation with flash number. This appears to be due primarily to a phase difference between oscillations calculated for high and for low Cl^- concentrations, which is not seen in the data. Such a difference could not be avoided in the simulations without assuming a highly cooperative Cl^- binding behavior that generates, for one K_d value, a strongly sigmoidal binding curve. Cooperativity is unlikely, however, given the observation that only one Cl^- is bound per PSII reaction center (*3*). In light of these findings, the assumption of a rapid binding equilibrium throughout the measuring flash train is untenable.

The conclusion that Cl^- exchange is neither slow nor fast as compared to the S-state cycle in these measurements has an important consequence. If no exchange occurred during measurement, the results would reflect Cl^- affinity in the

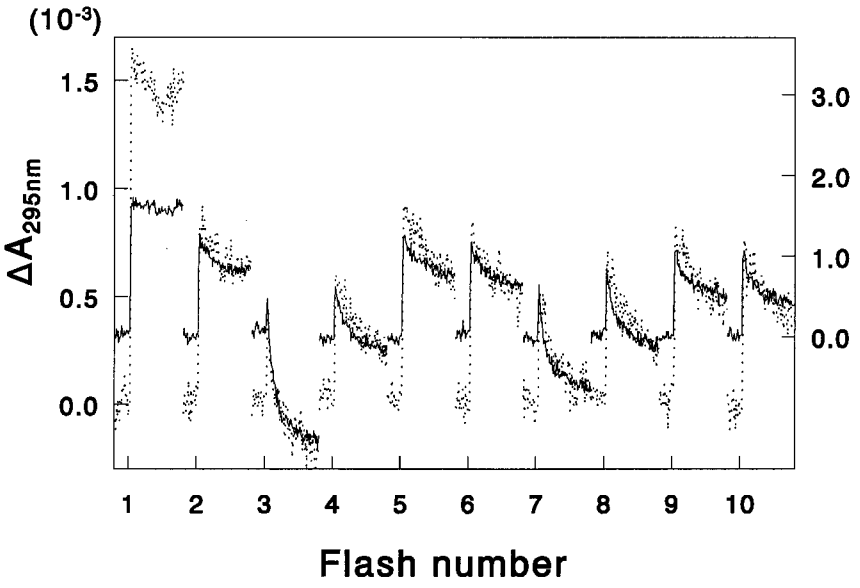

FIGURE 6: Absorbance changes at 295 nm scaled to reveal similar oscillation patterns. The Cl^- concentrations were 0.5 (dotted line, left scale) and 40 mM Cl^- (solid line, right scale). The change in the first flash is nearly Cl^--independent. The saturating flashes were spaced at 1 s.

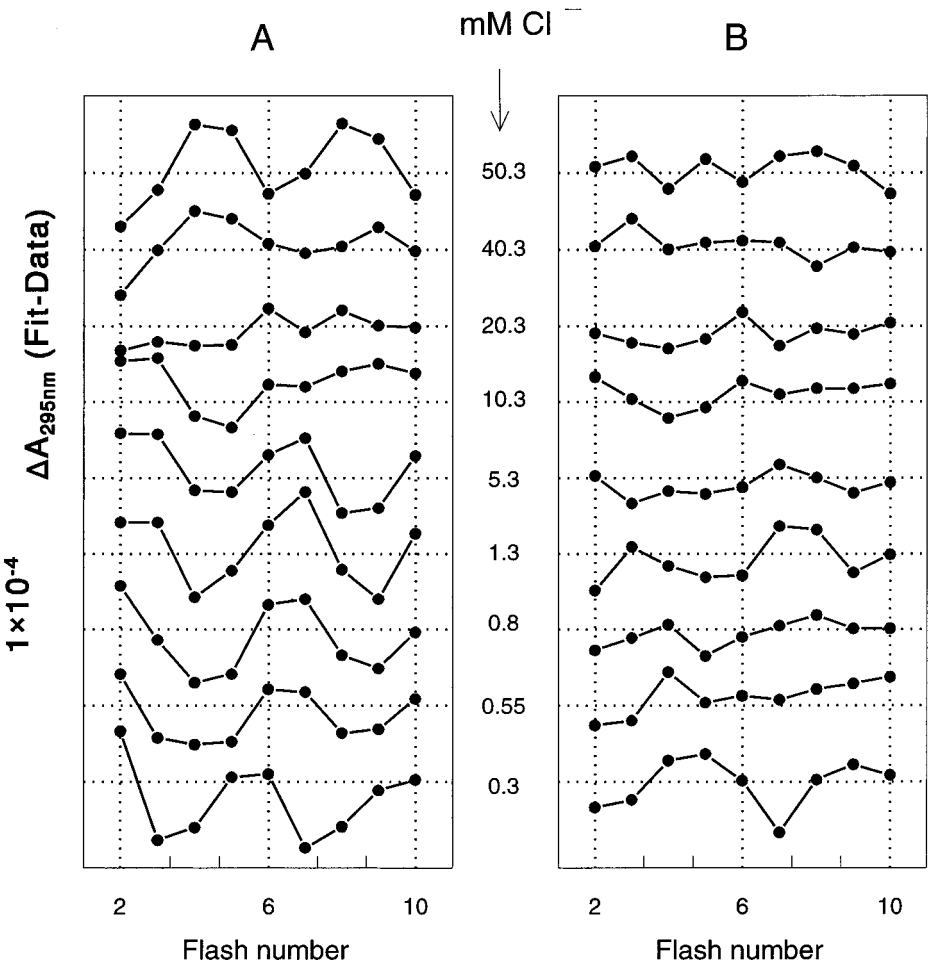

FIGURE 7: Residuals (fit minus data) from a global fit of the measurements of Figure 4 (triangles): (A) assuming equilibrium at a distribution of K_d values; (B) assuming a homogeneous, S-state-dependent K_d value and allowing incomplete Cl^- reequilibration between flashes.

dark-adapted state, as in intact PSII. If Cl^- binding were at equilibrium throughout the measurement, the results would reflect Cl^- affinity either in S_2 or in S_3, whichever is the lowest. Since Cl^- exchange in the time between flashes is obviously neither complete nor negligible, however, the assumption of constant binding properties may not be valid: if different S-states have different Cl^- affinities, binding equilibrium is never reached, and after each flash only partial reequilibration to the new K_d occurs before the next flash changes the K_d again. This model was implemented as

described under Materials and Methods, and the residuals from the simultaneous fit of the same data set as in Figure 7A are shown in Figure 7B. Although the fit is clearly much better, the values obtained for the Cl^- binding properties of each of the S-states could not be determined with sufficient precision on the basis of these data alone.

To further test this model and determine more accurately the Cl^- affinities and exchange rates in higher S-states, experiments were carried out with one or two preilluminating flashes in the presence of varying amounts of Cl^- that were added before or after the preilluminating flashes. The resulting simultaneous fit of all data is shown in Figure 8, and the values obtained for the equilibrium constant K_d and the time constant τ_d of Cl^- dissociation are listed in Table 1, left column (pH 7.5). In these simulations, the values for S_0 appeared to have little influence on the fit, probably because Cl^- is not required until the $S_2 \rightarrow S_3$ transition. Therefore, the S_0 values were set equal to those for S_1 in the fit shown. The lower and upper limits given between parentheses in Table 1 were estimated by constraining each variable to higher or lower values until the best global fit became obviously unacceptable for some of the individual measurements (when the root-mean-square difference between fit and data exceeded 2 times the rms noise in the data). These imposed changes were compensated for principally by changes in the other K_d and τ_d values, and led to minor changes in the other free parameters: dark distribution of S-states, extinction coefficients, which were found to be the same as reported by van Leeuwen et al. (*41*), misses, actinic effect of the measuring light. Taking all of these parameters into account, it can be concluded that a model proposing an intermediate rate of Cl^- exchange can explain the data only if K_d is higher in S_2 than in S_1, probably by an order of magnitude, and that the K_d is probably even higher in S_3 than in S_2.

The results just described were obtained at pH 7.5; in view of the pH dependence of Cl^- affinity (*42*), these data cannot be compared directly to those from intact PSII at pH 6, reported above. To obtain comparable data at pH 6.0, PSII membrane fragments were Cl^-/polypeptide-depleted by pH 7.5/sulfate treatment, pelleted by centrifugation, and resuspended in 25 mM MES, pH 6.0, 0.4 M sucrose. Flash-induced 295 nm absorbance changes after addition of various Cl^- concentrations were recorded and analyzed by computer simulations as above. Again no distribution of K_d values was found that could provide a reasonable fit of the data if rapid Cl^- exchange was assumed. The model allowing S-state-dependent Cl^- exchange in seconds led to the results in Table 1, right column. The K_d values in S_2 and especially S_1 were lower than at pH 7.5. Despite the absence of the 17 and 23 kDa extrinsic polypeptides and resulting rapid Cl^- exchange dynamics, the best-fit K_d in S_1 at both pH values is even slightly lower than that reported by Homann (*42*) for steady-state O_2 evolution by intact PSII.

DISCUSSION

Our results confirm and extend the characterization of the NO_3^- effect on the kinetics of the $S_3 \rightarrow S_0$ transition first reported by Sinclair (*32*), and show that this phenomenon depends only on the presence of NO_3^- in the Cl^- binding site. The fact that the NO_3^--supported $S_3 \rightarrow S_0$ transition is 3 times slower than that obtained with Cl^- indicates that the occupant of the PSII Cl^- site has a direct role in poising the activation energy of the rate-limiting step in the reduction of $Y_Z^{\bullet}S_3$ to Y_ZS_0. The kinetic effect elicited by NO_3^- is also, as we show here, a convenient probe of exchange dynamics at the Cl^- binding site. The 3-fold difference in $S_3 \rightarrow S_0$ kinetics is large enough to permit accurate determination of the amplitudes of the Cl^-- and NO_3^--dependent kinetic components of a mixed trace provided that the kinetics for 100% Cl^- and 100% NO_3^- are precisely measured in the same preparation under the same conditions, as is shown in the data of Figure 1.

The data of Figure 2 show that although high (millimolar) NO_3^- concentrations are required for the exchange process, Cl^- can be replaced more rapidly than has been reported (1 h) for its release in the S_1 state when the medium contains little or no Cl^- or competing anions (*3*). Since the OEC affinities for NO_3^- and Cl^- seem to be quite similar, the observation that relatively high NO_3^- concentrations are required to obtain facile exchange suggests that this process takes place in a sequestered domain, which communicates with the bulk medium through a large diffusion barrier. Such a domain would have to be enclosed by PSII itself, because PSII membrane fragments do not form closed vesicles. The volume of this space is therefore unlikely to exceed a few cubic nanometers, and given the reported K_d value (20 μM) in S_1 (*3*), the probability of finding a Cl^- in such a volume, if there were no interactions with its boundary, would be on the order of 0.01%. This consideration suggests, first, that when a Cl^- is trapped behind the barrier, it occupies its binding site virtually continuously and, second, that even a large increase in the Cl^- K_d value would increase proportionally the exchange rate with the bulk medium. The observation that substitution of NO_3^- for Cl^- appeared to be about 20 times faster in the S_2 and S_3 states than in S_1 (Figure 3) might be due to a corresponding increase in K_d, but the short lifetimes of S_2 and S_3 preclude experimental verification of this possibility in the presence of the diffusion barrier.

Dissociation of the extrinsic 17 and 23 kDa polypeptides removes or greatly reduces the Cl^- diffusion barrier, since the time required for Cl^- exchange with the bulk medium decreases from about an hour to seconds. However, this exchange is still slow in comparison to the rate of S-state turnover. The rate of Cl^- exchange is too slow to reach an equilibrium in the dark intervals between flashes, and rate constants for Cl^- binding and dissociation in each of the individual S-states were obtained from computer simulations that give a satisfactory fit of the data. Such simulations require eight variables, and changes in one variable can be compensated to some extent by adjustment of the other variables. For some variables, most notably the K_d values in S_1 and S_2 and the dissociation time in S_2, the allowed range of values is sufficiently narrow to allow definite conclusions. First, rapid Cl^- exchange in the S_2 and S_3 states in the (sub)millisecond time domain, which would be required to explain the NMR results of Preston and Pace (*23*), is not confirmed. Second, the K_d is higher in S_2 than in S_1, and at pH 6.0 this difference might well be large enough to account for the approximately 20-fold higher rate of NO_3^- substitution estimated for intact PSII on the basis of the data in Figure 3. Third, the K_d value in S_1 and its pH dependence correspond with the values reported for steady-

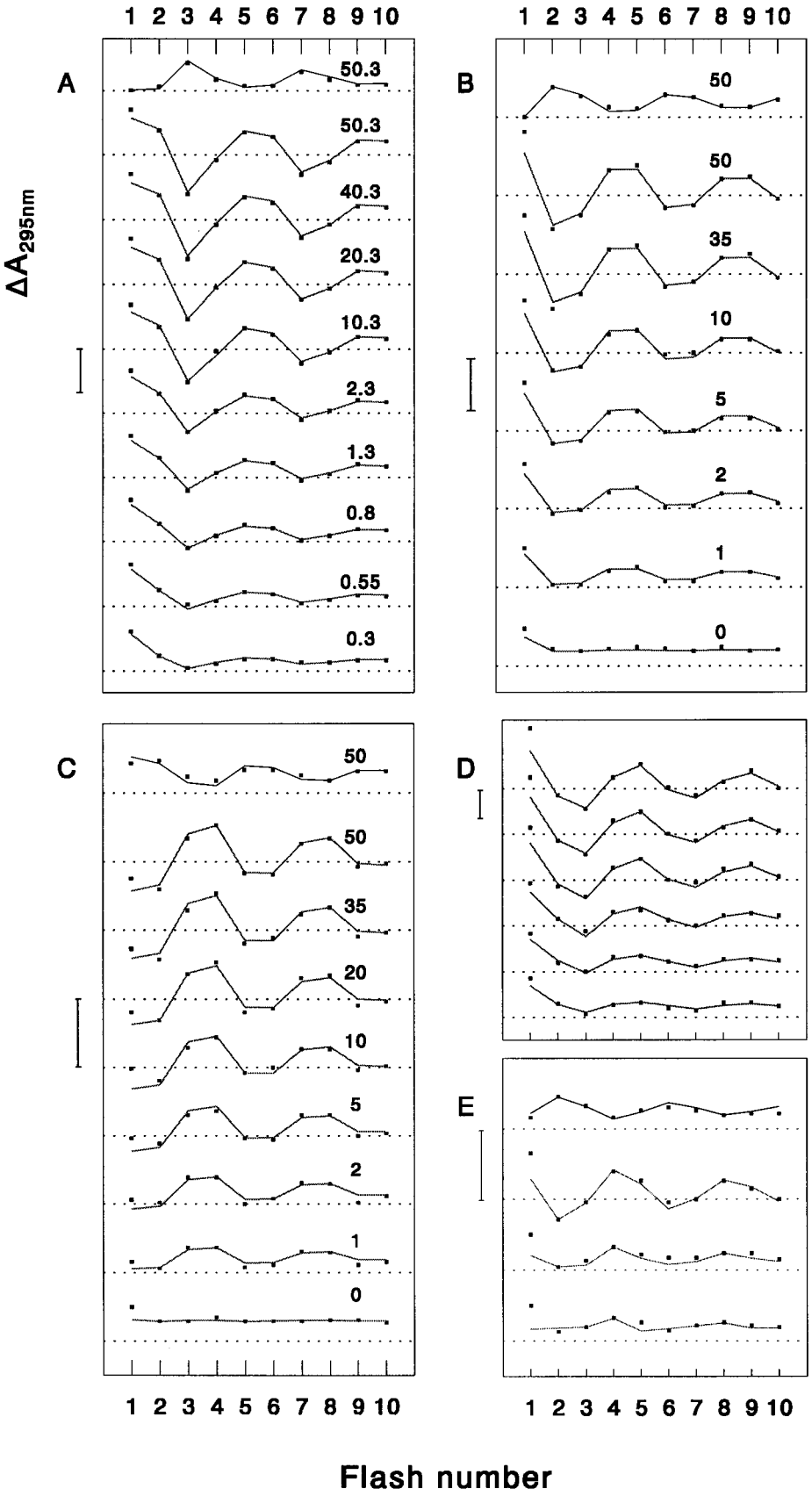

FIGURE 8: Simultaneous fit as in Figure 7B of 295 nm absorbance changes at different Cl^- concentrations, including data obtained in various Cl^- addition/preillumination conditions. Model as in Figure 7B. (A) Dark-adapted samples (the same data as previous figures); (B) 15 s after one preilluminating flash; (C) 15 s after two preilluminating flashes; (D) as (B) but Cl^- added after preillumination; (E) as (C) but Cl^- added after preillumination. Top traces in each frame except (D) represent fits of the absorbance transients due to $S_3 \rightarrow S_0$, shown at half-amplitude (corresponding to their weight in the fit). The vertical line beside each of the frames represents $\Delta A = 1 \times 10^{-3}$.

Table 1: Best-Fit Values and Maximum Range of the Cl^- Dissociation Equilibrium Constant K_d and the Dissociation Time Constant τ_d in PSII Depleted of Cl^- and the Extrinsic 17 and 23 kDa Extrinsic Polypeptides by pH 7.5/Sulfate Treatment

		pH 7.5	pH 6.0
K_d (mM)	S_0	nd	n.d.
	S_1	1.0 (0.6−2.0)	0.08 (0.01−0.2)
	S_2	7 (3−10)	1.0 (0.2−5)
	S_3	45 (>0.5)	130 (>0.5)
τ_d(s)	S_0	nd	nd
	S_1	10 (<10*)	nd
	S_2	11 (5−40)	2.5 (1−15)
	S_3	35 (>10)	2 (0.2−20)

[a] The limits shown were obtained with all other parameters unconstrained. Other fit parameters were the following: misses, 6%; hits due to measuring light, 5.7%; dark S_0, 25.4%; relative extinction coefficients: $S_0 \rightarrow S_1$, 1.9; $S_1 \rightarrow S_2$, 6.0; $S_2 \rightarrow S_3$, 4.2; $Y_Z \rightarrow Y_Z^{\bullet}$, 2.6, and offset (presumed to arise from the acceptor side), 1.0. The upper limit for τ_d in S_1 at pH 7.5 was imposed on the basis of the data shown in Figure 5; its value at pH 6.0 and all values for the S_0 state appeared to have little effect on the fit.

state rates of O_2 evolution by intact PSII. Therefore, removal of the extrinsic 17 and 23 kDa polypeptides does not seem to substantially change the affinity of the Cl^- binding site. We emphasize that this conclusion does not contradict the common notion that O_2 evolution requires much higher Cl^- concentrations in the absence of the 17 and 23 kDa polypeptides under steady-state illumination. The much higher K_d values we have determined for the higher S-states will lead to temporary inactivation by loss of Cl^- in a matter of seconds, and the resulting steady-state rate of O_2 evolution will be governed by the lower Cl^- affinities of S_2 and S_3. In the presence of the extrinsic proteins, a much longer time would be required to reach the same steady-state, so the S_1 Cl^- affinity should be dominant under this condition.

In Cl^- replacement experiments, in intact PSII, the exchange rate depends on the concentration of the added anion, and the process can occur more rapidly than does the release of Cl^- into a Cl^--free medium. Once a substituting anion has penetrated the polypeptide barrier, both ions would alternate with one another in spending a substantial fraction of time in the same sequestered domain, if they have similar K_d values for the binding site, because the binding site itself accommodates only one anion. Assuming a domain volume of at most few cubic nanometers, both ions will be present at an effective minimum concentration of about 0.1 M until one of the two is lost to the external medium, which will happen very quickly as a result of the large concentration gradient.

Our conclusion that the Cl^- affinity is changed substantially by the oxidation of Mn^{III} to Mn^{IV} on the $S_1 \rightarrow S_2$ transition supports the hypothesis that Cl^- is a Mn ligand (*17*), although conclusive spectroscopic evidence in support of this hypothesis is yet to be obtained. Since the ligand field stabilization energy would increase on oxidation of Mn^{III} to Mn^{IV}, the affinity decrease we detect in our experiments is more likely due to steric constraints arising from the size difference between Cl^- (1.7 Å) and Mn^{IV} (0.5 Å) (*22*).

On earlier occasions, precise simulations of S-state turnover in a flash series have been obtained by assuming a reversible temporary inactivation of the S_3 state (*41*, *43*). On the basis of the present data, it seems likely that the Cl^- concentration in those measurements was near or below the K_d in the S_3 state and the model used here would fully account for those data. Such conditions may be common both in experimental and in physiological circumstances, suggesting that the extrinsic polypeptides play an essential role in retarding the inevitable loss of Cl^- from PSII during illumination.

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