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Activating Anions That Replace Cl^- in the O_2 -Evolving Complex of Photosystem II Slow the Kinetics of the Terminal Step in Water Oxidation and Destabilize the S_2 and S_3 States[†]

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ABSTRACT: Photosystem II, the multisubunit protein complex that oxidizes water to O_2 , requires the inorganic cofactors Ca^{2+} and Cl^- to exhibit optimal activity. Chloride can be replaced functionally by a small number of anionic cofactors (Br^- , NO_3^- , NO_2^- , I^-), but among these anions, only Br^- is capable of restoring rates of oxygen evolution comparable to those observed with Cl^- . UV absorption difference spectroscopy was utilized in the experiments described here as a probe to monitor donor side reactions in photosystem II in the presence of Cl^- or surrogate anions. The rate of the final step of the water oxidation cycle was found to depend on the activating anion bound at the Cl^- site, but the kinetics of this step did not limit the light-saturated rate of oxygen evolution. Instead, the lower oxygen evolution rates supported by surrogate anions appeared to be correlated with an instability of the higher oxidation states of the oxygen-evolving complex that was induced by addition of these anions. Reduction of these states takes place not only with I^- but also with NO_2^- and to a lesser extent even with NO_3^- and Br^- and is not related to the ability of these anions to bind at the Cl^- binding site. Rather, it appears that these anions can attack higher oxidation states of the oxygen evolving complex from a second site that is not shielded by the extrinsic 17 and 23 kDa polypeptides and cause a one-electron reduction. The decrease of the oxygen evolution rate may result from accumulated damage to the reaction center protein by the one-electron oxidation product of the anion.

Photosystem II (PS II) has the unique ability to use water as an electron donor. The coupling of the one-electron photochemical events at the reaction center to four-electron water oxidation chemistry is achieved by cooperation of several polypeptides of PS II with inorganic components of the enzyme system. A central role in the water oxidizing activity of the oxygen evolving complex (OEC) is assigned to a tetranuclear Mn cluster, but in addition one atom each of Ca^{2+} (1) and of Cl^- (2) are required for the reaction to occur. The accumulation of the four oxidizing equivalents in the OEC and their subsequent release in water oxidation chemistry is described by the so-called S-state cycle proposed by Kok et al. (3). Each S_n (n is 0–4) state represents an oxidation state of the donor side of PS II, the subscript indicating the number of accumulated oxidizing equivalents.

Removal of Cl^- from PS II results in a block of the donor side reactions after formation of an S_2 state that is abnormally stable (4) and not accompanied by the formation of the $g =$

2 EPR multiline signal (5, 6). We have shown that the presence of Cl^- is necessary only for the $\text{S}_2 \rightarrow \text{S}_3$ and $\text{S}_3 \rightarrow \text{S}_0$ transitions of the OEC, while the rest of the cycle can proceed in its absence (7). This pattern for the Cl^- requirement of the OEC may be rationalized in terms of a model where Cl^- is required as a ligand to Mn so as to allow electron transfer between different Mn atoms in the tetranuclear cluster (7). More information on the role of Cl^- in affecting Mn redox chemistry may be obtained by studying the effects of substituting different anions for Cl^- , where the chemical properties of such ions (ionic radius, hardness, pK_a , and redox activity) might influence the activity of the OEC. If Cl^- is indeed a Mn ligand, as proposed by Sandusky and Yocum (8), one should not only expect the binding properties of activating anions to depend on the Mn oxidation state, which we recently confirmed (9), but conversely also the properties of the Mn cluster and its electron-transfer reactions may depend on the anion that occupies the Cl^- site.

The replacement of Cl^- by other anions supporting oxygen evolution has been studied by many authors (8, 10–12). Br^- appears to work as well as Cl^- and has been shown to compete with Cl^- for the same binding site (2). NO_3^- binds to the site with an equivalent affinity (2), but the maximum rate of oxygen evolution supported by NO_3^- is much lower and the kinetics of the $\text{S}_3 \rightarrow \text{S}_0$ transition is markedly slower than that observed in the presence of Cl^- (9, 13). Iodide also

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Table 1: Effects of Anion Addition on the Steady-State Rates of Oxygen Evolution, on the Kinetics of the $S_4 \rightarrow S_0$ Transition, and the Initial Activity of the OEC upon Flash Illumination of Dark-Adapted Samples as in Figures 1 and 2^a

	Cl ⁻	Br ⁻	NO ₃ ⁻	NO ₂ ⁻	I ⁻	F ⁻	none
$\mu\text{mol of O}_2 \times (\text{mg of Chl})^{-1} \times \text{h}^{-1}$	256	225	98	44	27	0	0
$S_4 \rightarrow S_0$							
τ_1 (ms)	3.3	4.9	15.6	8.2	11.7		
τ_2 (ms)	15.0	19.0	31.0	24.2	26.7		
A_1 (%)	53.9	40.8	54.1	44.1	46.3		
$\Delta A_{6-3+6-7} (10^{-3})$	3.01	2.74	1.94	1.56	1.95	0.13	0.33

^a The decays associated with the $S_4 \rightarrow S_0$ reaction were found to be biexponential for all anions tested, and the relative contribution of the fast phase is shown as A_1 . As a measure of the oscillation amplitude the difference between absorbance changes on flash numbers 6 and 3 plus that between 6 and 7 is given (compare Figure 2).

appears to support oxygen evolution, but its low reduction potential ($\text{I}^- \rightarrow {}^1/2\text{I}_2 + \text{e}^-$; $E^\circ = 0.535 \text{ V}$ (14)) should make this anion susceptible to oxidation by PS II. Such a reaction appears, in fact, to be responsible for a specific iodination of the secondary electron donor Y_Z , Tyr_{161} of the D_1 protein, especially in PS II particles from which the extrinsic 17 and 23 kDa polypeptides have been removed without a concomitant release of the functional Mn cluster (15). Addition of Cl^- to the iodination reaction mixture completely prevented iodination of the D_1 polypeptide, which suggests that the electron donor activity of I^- is exerted from the site where cofactor anions activate the OEC. This conclusion was brought into question, however, by later results of Papagiorgiou and Lagoyanni (16), who reported that I^- interfered noncompetitively with the reactivation of salt-washed PS II by Cl^- , and by results of Rashid and Homann (17), who concluded that I^- could replace Cl^- and support high rates of oxygen evolution, provided that the anion is bound only at the cofactor site in PS II and is not present in the bulk medium; i.e., I^- bound at the Cl^- site appeared to be immune to oxidation.

In this article we report the result of experiments in which UV spectroscopy was used to analyze in detail the effects of a series of surrogate anions on electron-transfer events at the donor side of photosystem II. An examination of the $S_4 \rightarrow S_0$ reaction (3), which we have earlier shown to require the presence of Cl^- (7), reveals that each of the anions that functionally substitute for Cl^- supports a different rate of the $S_4 \rightarrow S_0$ reaction. In all cases, this transition was slower with surrogate anions than with the native cofactor, Cl^- . In addition, all anions tested caused a reduction of the S_2 and S_3 states, but this effect is not related to the substitution of the anion for Cl^- at the activator binding site.

MATERIALS AND METHODS

Photosystem II membrane fragments from spinach were prepared as described by Berthold et al. (18) with modifications (19) and stored at 3–4 mg Chl/mL at 77 K in buffer containing 20 mM MES (pH 6.0) and 0.4 M sucrose.¹ These preparations are referred to as “untreated PS II membrane fragments”. Chloride and the 17 and 23 kDa extrinsic polypeptides were removed by a 15 min incubation of PS II membranes 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 chlorophyll concentration of 0.2 mg/mL. The UV measurements were performed directly after incubation and addition of Cl^- or surrogate anions (Na salts, concentrations given in the text). The lifetimes of higher S-states were determined in untreated PS II membrane fragments, using buffer containing 20 mM MES pH 6.0 and 0.4 M sucrose, in the presence of Cl^- alone or in combination with another anion (concentrations are given in the text). The artificial electron acceptors DCBQ and ferricyanide (final concentrations: 50 and 100 μM , respectively) were added to avoid limitations from the PS II acceptor side. The residual Cl^- contamination of buffers was determined as described by Lindberg et al. (2) and was routinely found to be in a range of 20–40 μM .

Absorbance difference measurements were performed at room temperature as described previously (20). Flash repetition rates are given in the figure legends. Steady-state rates of oxygen evolution were assayed with a Clark electrode at 20 °C, in the presence of 2.5 mM ferricyanide and 0.4 mM DCBQ at a Chl concentration of 20 $\mu\text{g/mL}$. In this case the Cl^- -depletion procedure was shortened to 1 min; this time was sufficient to completely inhibit oxygen evolution, probably due to the lower Chl concentration.

Computer simulations of the S-state turnover according to the Kok cycle (3) were performed using the NAG Library routine E04JAF for optimizing parameter values. Data used for fitting the S-state turnover at pH 7.5 consisted of stable absorbance changes, measured 80 ms after the flash in case of Cl^- , Br^- , and I^- and 130 ms after the flash in case of NO_3^- and NO_2^- . The first flash was disregarded to avoid complications from nonoscillating absorbance changes due to inactive centers (21). To determine the S_2 and S_3 lifetimes untreated PS II membranes were illuminated with series of saturating flashes while varying the time between the 1st and the 2nd flash (S_2) or between the 2nd and the 3rd flash (S_3). The resulting oscillation patterns (stable absorbance changes, 40 ms after the flash) were used to fit the decay time of the higher S-states.

RESULTS

When PS II membrane fragments are depleted of Cl^- by pH 7.5 sulfate treatment, the resulting preparations lack the 23 and 17 kDa extrinsic proteins and do not advance beyond the S_2 state (7). Oxygen evolution could be restored in these preparations by readdition of Cl^- itself or by addition of one of the following anions: Br^- , NO_3^- , NO_2^- , or I^- . The rates of oxygen evolution are shown in Table 1 and agree with results published elsewhere (8, 12) for preparations that retain the extrinsic polypeptides.

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'*-ethanesulfonic acid; Y_Z , the tyrosine residue that acts as a primary electron donor to P_{680} .

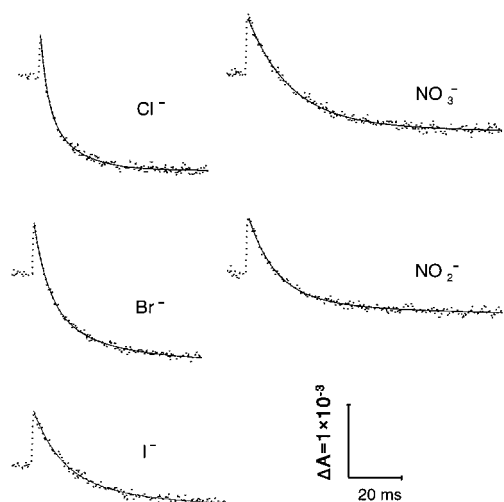


FIGURE 1: Absorbance changes at 295 nm induced by the third flash in PS II membrane fragments reconstituted with Cl⁻, Br⁻, NO₃⁻, NO₂⁻, and I⁻. The measurements were performed at pH 7.5, after addition of the Na salts of the anions to Cl⁻-depleted samples. The anions were added to a final concentration of 50 mM, and the excitation flashes were spaced at 1 s, except in the case of I⁻, where a concentration of 10 mM and a flash spacing of 100 ms were used.

To gain more information about the effects that Cl⁻ surrogates exert on the individual S-state transitions, measurements were performed utilizing flash-induced UV absorbance difference spectroscopy. At 295 nm, where the contribution of absorbance changes from the acceptor side is minimal (22), the donor side reactions induced by a series of 10 saturating flashes were measured. The most striking effects were observed in the kinetics of the signals induced by the third and following flashes, which appeared to be different for each of the anions used for reconstitution.

The signals measured on the third flash after reconstitution with Cl⁻, Br⁻, I⁻, NO₃⁻, and NO₂⁻ are shown in Figure 1. The initial absorbance increase is due to the S₂ → S₃ transition in centers missed on the first or second flash and to the oxidation of Y_Z in centers that were in S₃. The resulting S₃Y_Z[•] state, which we will refer to as S₄, reacts with water and reverts to S₀, causing an absorbance decay in a few milliseconds. For all added anions the absorbance decayed with biexponential kinetics. The parameters that yielded the best fit to the data are listed in Table 1. Such biexponential decays are also observed in untreated PS II membrane fragments at pH values above 7 (data not shown). In contrast, at pH 6.0 the S₄ → S₀ decays were always monophasic and more rapid than the fast phase at pH 7.5 (9). The appearance of the slow phase at high pH coincides with that of trypsin sensitivity, attributed to a conformational change controlled by deprotonation of a group with pK_a = 7.6 (23). It could be reversed after removing the 23 and 17 kDa proteins by centrifugation, but the experiments shown here were done directly at pH 7.5 to minimize both the activity decrease associated with further sample handling and the acceleration of S-state decay discussed below. In particular the fast phase shows a pronounced dependence on the anion occupying the cofactor site. This phase is slowest after addition of NO₃⁻, but even Br⁻, an anion that supports rates of oxygen evolution and oscillations in the absorbance changes that are very similar to those obtained in a system reconstituted with

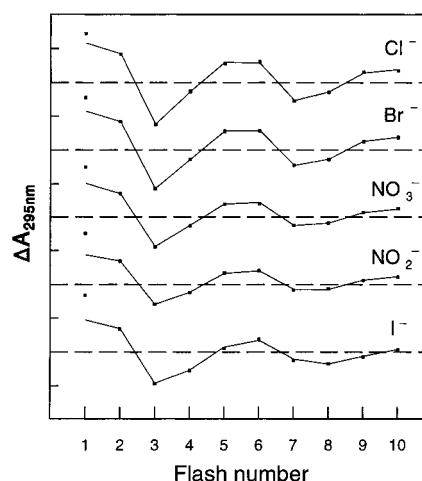


FIGURE 2: Fit of the 295 nm absorbance changes induced by 10 saturating flashes in PS II after reconstitution with various anions. The anion concentrations and the flash frequency were the same as in Figure 1. The datapoints indicate the final level of the absorbance change, read at 80 or 130 ms after the flash (see Methods). The fit parameters were the following: misses, 4.1% (in the case of I⁻ the miss factor was 11.3%, probably due to incomplete reoxidation of Q_A⁻ at the higher flash frequency); hits due to measuring light, 2.4%; relative extinction coefficients: S₀ → S₁, 1.9; S₁ → S₂, 6.0, S₂ → S₃, 4.2, and Y_Z → Y_Z[•], 2.6; offset, 0.16 (probably due to the acceptor side). The initial amount of S₀ was 13.1%, except for NO₂⁻ (20.1%), and the values required for temporary inactivation of S₃ to fit the oscillation patterns shown were as follows: 31.5% (Cl⁻); 31.4% (Br⁻); 42.3% (NO₃⁻); 33.9% (NO₂⁻); 36.2% (I⁻). Deactivation of the S₃ state to S₂ between flashes was 14.5% for NO₂⁻, and it was negligible for the other anions. In the presence of I⁻, deactivation of the S₃ state was minimized by the lower concentration of the anion and by the higher flash frequency. The distance between the dashes lines (zero lines) represent ΔA = 2 × 10⁻³.

Cl⁻, causes a change in S₄ → S₀ decay kinetics that is not insignificant.

The signals shown in Figure 1 differ not only in their kinetics but also, rather significantly, in their amplitudes. By computer simulations of our data more information could be gained about the S-state turnover in the presence of various Cl⁻ surrogates. The data shown in Figure 2 are the oscillation patterns of the "stable" absorption changes, at 80 or 130 ms after each flash, obtained for anions specified in the figure. Clearly the reconstitution of the first S-state cycle under single-turnover condition is much better than that of steady-state O₂ evolution with NO₃⁻, NO₂⁻, or I⁻. By the same method no oscillation could be restored with F⁻ (Table 1). The data show significant differences in the oscillatory behavior, depending on the anion present in the sample. The parameters required to fit the period-four Kok model to these oscillations are presented in the legend to the figure. It was possible to obtain good fits for all samples by assuming the existence of a temporary inactivation of the S₃ state (24). This inactivation was found to be similar for Cl⁻ and Br⁻, slightly higher for NO₂⁻ and I⁻ and highest for NO₃⁻, but the differences are small. We have recently shown that the affinity of the Cl⁻ binding site is strongly S-state dependent and proposed that dissociation of the anion in the S₃ state may well explain the success of the assumption of a reversible inactivation of the S₃ state in modeling S-state turnover kinetics (9). Different extents of inactivation with different Cl⁻ surrogates would then reflect differences among these anions with respect to their binding properties in the

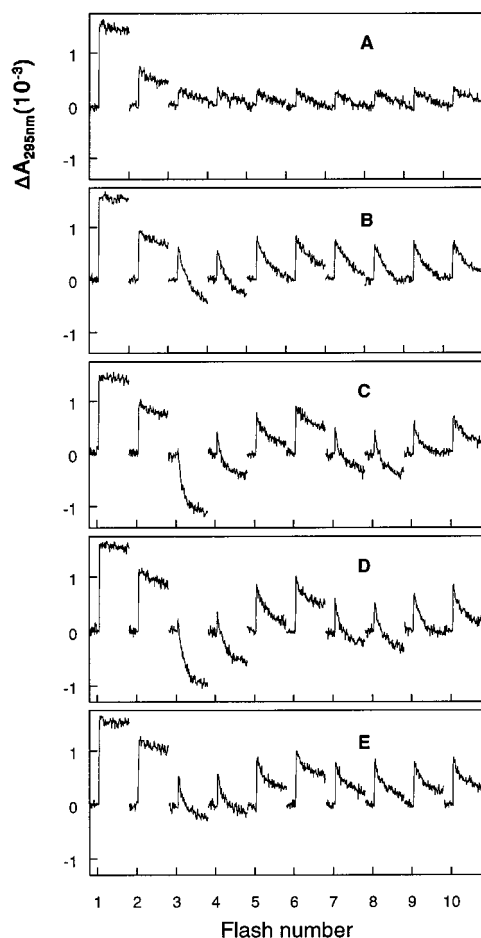


FIGURE 3: Absorbance changes at 295 nm induced by 10 saturating flashes in the following: Cl^- -depleted PS II (A); PS II reconstituted with 10 mM NaI (B); PS II reconstituted with 50 mM NaCl (C); PS II reconstituted with 50 mM NaCl and 10 mM NaI (D and E). The flashes were spaced at 100 ms except for the last measurement (E), where the flash frequency was 1 Hz. Each trace represents 50 ms.

S_3 state. Alternatively, the slower $\text{S}_4 \rightarrow \text{S}_0$ kinetics might cause extra misses on this transition due to charge recombination of $\text{Q}_\text{A}^-\text{S}_4$, but this assumption did not improve the fits. In the case of I^- and NO_2^- , however, a rapid deactivation of S_3 to S_2 had to be assumed. With 50 mM of the anion and a flash repetition rate of 1 Hz, 14.5% of S_3 decayed to S_2 between flashes in the case of NO_2^- and no oscillation could be detected in the case of I^- . At 10 mM I^- and a 10 Hz flash repetition rate, however, pronounced oscillations were observed, indicating that now S_3 to S_2 decay was negligible. The kinetics in such conditions are shown in Figure 3B, together with a measurement without (A) and with 50 mM Cl^- (C), at the same flash frequency. Although with 10 mM I^- the synchronization of the cycle is lost more rapidly and the $\text{S}_4 \rightarrow \text{S}_0$ transition is slower, the average amplitude of the absorbance decrease associated with this transition on the first 10 flashes is not much smaller than the amplitude observed in the presence of 50 mM Cl^- . Since the oxygen yield is proportional to the extent of this absorbance decrease (25), the oxygen yield of the first 10 flashes is similar with both anions. The much lower steady-state rate of I^- -supported O_2 evolution in continuous light (Table 1) may be due to gradual inactivation of the sample as a result of the high number (hundreds) of turnovers involved in the steady-state measurement. This is very likely

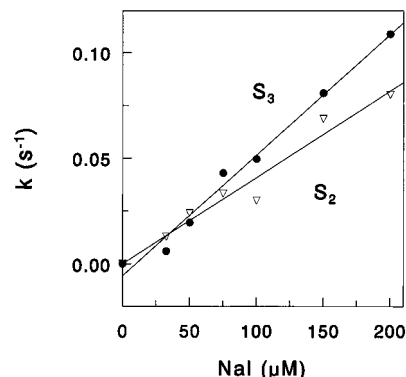


FIGURE 4: Dependence of the initial rate of reduction of the S_2 and S_3 states on the I^- concentration in untreated PS II membranes at pH 6.0, in the presence of 30 mM NaCl. The samples were preilluminated with one or two flashes, and 10 s later the measurement followed (a series of 10 flashes). The absorbance changes at 40 ms after each flash were used to fit the decay rates of the S_2 and S_3 states. The k values presented were obtained by subtracting the 'native' rate constants of the S-states reduction (shown in Table 1, column 'none').

to be the case. In the presence of I^- the O_2 evolution rates measured with a Clark electrode showed a rapid decline during illumination and measurement, while nearly straight lines indicative of robust steady-state activity were obtained with Cl^- (not shown). Presumably the damage results from the oxidation products of I^- , e.g. iodination of Y_2^* (15), from damage caused by long-lived radicals (P_{680}^+ , Y_2^*) or from both.

When both 10 mM I^- and 50 mM Cl^- were added simultaneously, the absorbance changes observed at 10 Hz flash frequency (Figure 3D) were not very different from those with Cl^- alone (Figure 3C). When the flash spacing was increased to 1 s, however, the effect of I^- in shortening the lifetime of the higher S-states was observed again (Figure 3E). The rapid $\text{S}_4 \rightarrow \text{S}_0$ kinetics in Figure 3D,E confirm that the anion binding site is largely occupied by Cl^- rather than by I^- , as in Figure 3B. This finding indicates that S-state reduction by I^- does not require access by I^- to the Cl^- binding site, in agreement with the finding that I^- bound at the Cl^- binding site does not appear to undergo oxidation (17, 26).

The measurements described so far were carried out after Cl^- depletion in 50 mM sulfate, pH 7.5, to obtain quantitative exchange of added anions at the Cl^- binding site. The presence of 50 mM sulfate was found to have no influence on these effects, but an effect of pH was observed: at pH 6.0 S-state reduction by I^- was about 10 times faster, in agreement with earlier results (15). This reaction was studied in more detail at pH 6.0 in untreated PS II membrane fragments, retaining the extrinsic 17 and 23 kDa proteins and Cl^- . To avoid significant substitution of surrogate anions for Cl^- at the activator binding site during the time required for the measurement of S-state reduction, 30 mM Cl^- was added to all incubation mixtures and the lifetimes of S_2 and S_3 were determined by fitting the 295 nm absorbance oscillation in a flash series, starting 10 s after preillumination. Figure 4 shows the dependence of the rate constants of S_2 and S_3 reduction on I^- concentration that were obtained using this procedure. The linear dependence on I^- concentration indicates that the formation of I_2 is not rate-limiting, if it happens at all; Izawa and Ort (27) were unsuccessful in

Table 2: Effect of Various Anions on the Lifetimes of the S₂ and S₃ States^a

	none	Br ⁻ (30 mM)	NO ₃ ⁻ (30 mM)	NO ₂ ⁻ (2.5 mM)	I ⁻ (100 μM)
τ(S ₂ → S ₁) (s)	159.5	87.1	51.1	121.7	27.5
<i>k</i>	6.2 × 10 ⁻³ s ⁻¹	0.17 M ⁻¹ s ⁻¹	0.44 M ⁻¹ s ⁻¹	0.77 M ⁻¹ s ⁻¹	301 M ⁻¹ s ⁻¹
τ(S ₃ → S ₂) (s)	73.3	19.6	17.8	15.4	15.8
<i>k</i>	1.36 × 10 ⁻² s ⁻¹	1.2 M ⁻¹ s ⁻¹	1.4 M ⁻¹ s ⁻¹	20.5 M ⁻¹ s ⁻¹	496 M ⁻¹ s ⁻¹

^a All measurements were performed using untreated PS II membranes at pH 6.0 in the presence of 30 mM NaCl. The "native" lifetimes and the corresponding decay rate constants of the S₂ and S₃ states in untreated PS II membranes, in the presence of Cl⁻ only, are shown in column "none". The τ values are the observed decay times of S₂ and S₃ states at the given concentration of active anion. The rate constants of S₂ and S₃ reduction (*k*) were calculated by subtracting the "native" rate constants and assuming that the anion-induced rate is proportional to the anion concentration.

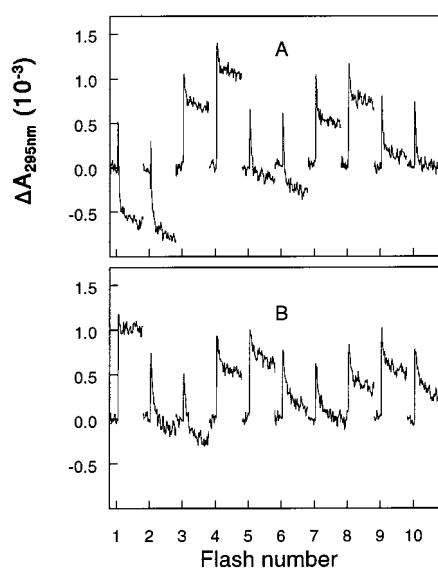


FIGURE 5: Oscillation pattern of the 295 nm absorbance changes in untreated PS II, recorded 30 s after 2 preilluminating flashes with 30 mM Cl⁻ present. In B the preilluminating flashes were followed by injection of NO₃⁻ to a final concentration of 30 mM.

demonstrating PS II catalyzed I₂ formation using methods of chemical detection, and we encountered similar difficulties (not shown). Moreover the pattern of S-state deactivation we have detected was clearly inconsistent with a two-electron reduction reaction. A direct reduction of the S₃ to S₁ state was not seen. These observations seem to eliminate I₂ and I⁺ as oxidation products of the deactivation of the higher S-states and suggest that I[•] is the product of the deactivation reaction.

Of the other surrogate anions investigated, only NO₂⁻ can be oxidized by S₃ at substantial rates, but all of these anions were found to be capable of destabilizing both S₂ and S₃ to some extent. This is illustrated for S₃ reduction by NO₃⁻ in Figure 5. In these experiments, a sample was given two preflashes in the presence of Cl⁻ to produce S₃. Then a surrogate anion was injected and mixed with the sample. After a dark period, the absorbance changes induced by a series of flashes were measured to determine the amount of S₃ still present in the sample at the end of the incubation period. As can be seen from the data in Figure 5, injection of NO₃⁻ clearly destabilized the S₃ state. The estimated lifetime of this state with NO₃⁻ was about 18 s, in contrast to the lifetime estimate in the presence of Cl⁻ only (ca. 73 s). The dark period between preilluminating flashes and NO₃⁻ injection should have been sufficient to permit complete reoxidation of the electron acceptors, which therefore do not

seem to be the electron source in the NO₃⁻ induced S-state reduction.

The data for all anions are summarized in Table 2. The lifetimes of S₂ and S₃ in the presence of Cl⁻ were independent of Cl⁻ concentration, which indicates that the presence of this anion does not produce any unusual destabilization of the higher S-states. In the calculation of the rate constant for S-state reduction by each of the anions examined, the decay rate observed with Cl⁻ alone was subtracted and the remaining rate was assumed to be proportional to the concentration of the other anion added to the system, as shown for I⁻ in Figure 4. None of the anions was found to reduce S₁, regardless of their ability to deactivate the higher S-states. Only in the case of NO₂⁻ a slightly enhanced S₀ concentration appeared to be present in the dark. This was more likely due to some Tyr_D reduction rather than to S-state reduction, since the absorbance change on the first flash was not diminished (Figure 2).

DISCUSSION

The results we report here show that, in PS II membrane fragments exposed to pH 7.5 and 50 mM sulfate, O₂ production in continuous light and flash-induced UV absorbance oscillations is strictly dependent on the addition of Cl⁻ or a surrogate anion. Cl⁻, Br⁻, I⁻, NO₂⁻, and NO₃⁻ can restore flash-induced UV oscillations and steady-state activity to varying extents, depending on the anion and its concentration, as well as on the type of measurement (single-turnover or steady-state) used to detect activity. Nitrite has not, to our knowledge, been previously shown to replace Cl⁻ as an activating anion, but its ability to do so is not surprising and has been suggested before (28). Maximum rates of oxygen evolution as measured with a Clark electrode decreased strongly in the order Cl⁻ > Br⁻ > NO₃⁻ > NO₂⁻ > I⁻, which is in accord with earlier findings (8, 10–12). The varying degrees of reconstitution with different anions suggested that a comparison of S-state turnover kinetics in relation to the properties of the individual anions might provide insights into the functional role of Cl⁻ in the OEC. As the data presented here show, reconstitution of the S-state cycle, as measured by UV absorbance changes induced by a series of 10 saturating flashes in a dark-adapted sample, did not exhibit a striking dependence on the nature of the added anion. Each of the anions reactivated the same number of PS II centers. The only property that was clearly dependent on the identity of the surrogate anion occupying the Cl⁻ binding site was the rate of the reduction of the S₄ state to S₀, the step that accompanies the oxidation of H₂O to O₂. The rate of this reaction decreased in the order Cl⁻ > Br⁻ > NO₂⁻ > I⁻ >

NO_3^- . For NO_3^- , such an effect on water oxidation kinetics has been well documented (9, 13), and Sinclair (personal communication) observed it also with NO_2^- . In the cases of Br^- and I^- the slower H_2O oxidation has not to our knowledge been previously observed, even though, as we show here, the difference between these ions and Cl^- is quite significant (Figure 1). Since Br^- and NO_3^- are equally effective in competing with Cl^- for the same binding site (2), their contrasting effects on the $\text{S}_4 \rightarrow \text{S}_0$ kinetics are unlikely to reflect differences in their binding affinities in the OEC. The anion dependence of the kinetics of the $\text{S}_4 \rightarrow \text{S}_0$ step suggests that these surrogates as well as Cl^- itself may play a role in poisoning the activation energy of the final step in the oxygen evolution mechanism, consistent with the hypothesis that Cl^- is a ligand to the Mn cluster (8). Like the halides, NO_3^- and NO_2^- are well documented as metal ligands (29); binding to Mn in the OEC would not be excluded by their size, 1.89 and 1.55 Å, respectively, which is actually smaller than the 1.97 Å of Cl^- (30).

The order of anions in exhibiting a kinetic effect on the $\text{S}_4 \rightarrow \text{S}_0$ reaction does not show any obvious correlation with ionic radius, hardness or softness of the anion, redox potential, or $\text{p}K_a$ (14, 31), which might further explain why Cl^- or a surrogate is required for the terminal step in the water oxidizing reaction.

Even with NO_3^- the $\text{S}_4 \rightarrow \text{S}_0$ reaction, at least as measured under single-turnover conditions, was too fast to be rate-limiting for steady-state O_2 evolution in continuous light, and extra misses on the $\text{S}_4 \rightarrow \text{S}_0$ transition due to charge recombination of S_4Q_A^- resulting from the longer lifetime of the S_4 state also did not seem to be responsible for the effects of the anions on the rate of O_2 evolution. In fact, the lower rates of O_2 evolution measured with a Clark electrode seem to correlate most closely with the ability of the anions to destabilize the higher S-states, which probably has nothing to do with the role of Cl^- in the OEC. This conclusion agrees with the observation of Rashid and Homann (17) that I^- supports high rates of oxygen evolution when its presence is restricted to the anion binding site.

Neither the extrinsic polypeptides nor the anion bound to the Cl^- site appeared to have a substantial influence on the anion-induced reduction of S_2 and S_3 . The exchange of NO_3^- for Cl^- at the cofactor binding site (1 h incubation of the untreated PS II membranes in the presence of 30 mM NO_3^-) had hardly any effect on the reduction rates of the S_2 and S_3 state by NO_3^- (not shown). The destabilization of the S_2 and S_3 states clearly takes place at a different site that does not seem to involve a long-lived association of the anion with the site; e.g. their reduction by I^- in the experiments of Table 2 does not seem to be impeded by the presence of 300-fold excess of Cl^- . An effect on the electron acceptor side seems unlikely because the S-state reduction took place long after reoxidation of the acceptor side by DCBQ and ferricyanide. It is tempting to speculate that the anions have access to the Mn cluster via the opening that allows $\text{H}_2\text{O}/\text{O}_2$ exchange although they may be too large to fit in the site of H_2O oxidation itself. On the other hand, the observed electron transfer rates do not necessarily imply a short distance between the anion and the Mn cluster (32), and in fact, although S-state destabilizations are consistent with their reduction by added anions, oxidation of the anions has not been demonstrated to occur. For I^- and NO_2^- , oxidation may

indeed be feasible, but it is not immediately obvious how PS II can withdraw an electron from NO_3^- (the estimated E° for the $\text{NO}_3^-/\text{NO}_3^\bullet$ couple in H_2O is reported to be 2.67 V (33)), although the generation of NO_3^\bullet radicals in solution has been reported to occur (34). The oxidation of halides such as I^- is normally a two-electron process that produces I_2 or HOI . The reduction potential for a one-electron oxidation could be much higher. Nevertheless, the observed S-state deactivation catalyzed by I^- is clearly a one-electron reduction reaction whose rate depends linearly on the I^- concentration. Perhaps one-electron oxidation of the halide is made possible energetically by a rapid reaction of the product radical with nearby amino acid residues. The resulting damage to the protein may explain the rapidly declining oxygen evolution rates.

REFERENCES

1. Ädelroth, P., Lindberg, K., and Andréasson, L.-E. (1995) *Biochemistry* 34, 9021–9027.
2. Lindberg, K., Vänngård, T., and Andréasson, L.-E. (1993) *Photosynth. Res.* 38, 401–408.
3. Kok, B., Forbush, B., and McGloin, M. (1970) *Photochem. Photobiol.* 11, 457–475.
4. Ono, T., Zimmermann, J. L., Inoue, Y., and Rutherford, A. W. (1986) *Biochim. Biophys. Acta* 851, 193–201.
5. Rutherford, A. W., Zimmermann, J.-L., and Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 179–229, Elsevier Science Publishers, New York.
6. Britt, R. D. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum C. F., Eds.) pp 137–164, Kluwer Academic Publishers, Dordrecht, The Netherlands.
7. Wincencjusz, H., van Gorkom, H. J., and Yocum, C. F. (1997) *Biochemistry* 36, 3663–3670.
8. Sandusky, P. O., and Yocum, C. F. (1984) *Biochim. Biophys. Acta* 766, 603–611.
9. Wincencjusz, H., Yocum, C. F., and van Gorkom, H. J. (1998) *Biochemistry* 37, 8595–8604.
10. Kelley, P. M., and Izawa, S. (1978) *Biochim. Biophys. Acta* 502, 198–210.
11. Homann, P. H. (1988) *Plant Physiol.* 88, 194–199.
12. Ono, T., Nakayama, H., Gleiter, H., Inoue, Y., and Kawamori, A. (1987) *Arch. Biochem. Biophys.* 256, 618–624.
13. Sinclair, J. (1984) *Biochim. Biophys. Acta* 764, 247–252.
14. Shriver, D. F., Atkins, P. W., and Langford, C. H., Eds. (1994) *Inorganic Chemistry*, Oxford University Press, New York.
15. Ikeuchi, M., Koike, H., and Inoue, Y. (1988) *Biochim. Biophys. Acta* 932, 160–169.
16. Papageorgiou, G. C., and Lagoyanni, T. (1991) *Arch. Biochem. Biophys.* 285, 339–343.
17. Rashid, A., and Homann, P. H. (1992) *Biochim. Biophys. Acta* 1101, 303–310.
18. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett.* 134, 231–234.
19. Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1984) *Biochim. Biophys. Acta* 765, 388–398.
20. Dekker, J. P., Van Gorkom, H. J., Brok, M., and Ouwehand, L. (1984) *Biochim. Biophys. Acta* 764, 301–309.
21. Lavergne, J., and Leci, E. (1993) *Photosynth. Res.* 35, 323–343.
22. Van Leeuwen, P. J., van Gorkom, H. J., and Dekker, J. P. (1992) *J. Photochem. Photobiol., B: Biol.* 15, 33–43.
23. Völker, M., Ono, T., Inoue, Y., and Renger, G. (1985) *Biochim. Biophys. Acta* 806, 25–35.
24. Van Leeuwen, P. J., Heimann, C., and van Gorkom, H. J. (1993) *Photosynth. Res.* 38, 323–330.

25. Van Leeuwen, P. J., Vos, M. H., and van Gorkom, H. J. (1990) *Biochim. Biophys. Acta* 1018, 173–176.
26. Homann, P. H. (1993) *Photosynth. Res.* 38, 395–400.
27. Izawa, S., and Ort, D. R. (1974) *Biochim. Biophys. Acta* 357, 127–143.
28. Sinclair, J. (1987) *Photosynth. Res.* 12, 255–263.
29. Cotton, F. A., and Wilkinson, G. (1980) *Advanced Inorganic Chemistry: A Comprehensive Text*, pp 173–174, John Wiley and Sons, New York.
30. Shriver, D. F., Atkins, P., and Langford, C. H. (1994) *Inorganic Chemistry*, 2nd ed., p. 173, W. H. Freeman and Co., New York.
31. Huheey, J. E. (Ed.) (1972) *Inorganic Chemistry: Principles of Structure and Reactivity*, Harper and Row Publishers, New York.
32. Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., and Dutton, P. L. (1992) *Nature* 355, 796–802.
33. Jiang, P. Y., Katsumura, U. Y., Ishigure, K., and Yoshida, Y. (1992) *Inorg. Chem.* 31, 5135–5136.
34. Neta, P., and Huie, R. E. (1986) *J. Phys. Chem.* 90, 4644–4648.

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