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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 $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ 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 $S_3 \rightarrow S_0$ 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 |
|---|---|---|---|---|---|---|---|
| $\mu$mol of $O_2$ × (mg of Chl)$^{-1}$ × h$^{-1}$ | 256 | 225 | 98 | 44 | 27 | 0 | 0 |
| $S_4 \rightarrow S_0$ | | | | | | | |
| $\tau_1$ (ms) | 3.3 | 4.9 | 15.6 | 8.2 | 11.7 | | |
| $\tau_2$ (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 ($I^- \rightarrow {}^1/_2 I_2 + e^-$; $E° = 0.535$ 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 $\mu$M, 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 $\mu$M.

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 $\mu$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.

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

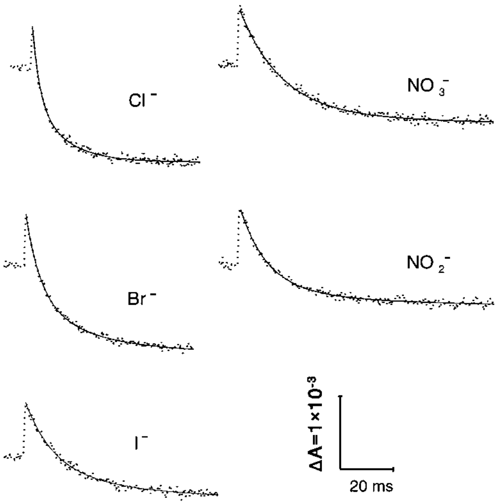

FIGURE 1: Absorbance changes at 295 nm induced by the third flash in PS II membrane fragments reconstituted with $Cl^-$, $Br^-$, $NO_3^-$, $NO_2^-$, and $I^-$. The measurements were performed at pH 7.5, after addition of the Na salts of the anions to $Cl^-$-depleted samples. The anions were added to 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 $Cl^-$, $Br^-$, $I^-$, $NO_3^-$, and $NO_2^-$ are shown in Figure 1. The initial absorbance increase is due to the $S_2 \rightarrow S_3$ transition in centers missed on the first or second flash and to the oxidation of $Y_Z$ in centers that were in $S_3$. The resulting $S_3Y_Z^\bullet$ state, which we will refer to as $S_4$, reacts with water and reverts to $S_0$, 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 $S_4 \rightarrow S_0$ 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 $NO_3^-$, 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 $Cl^-$, causes a change in $S_4 \rightarrow S_0$ decay kinetics that is not insignificant.

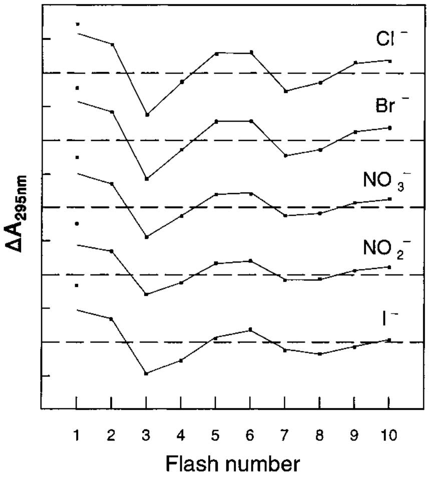

FIGURE 2: Fit of the 295 nm absorbance changes induced by 10 saturating flashes in PS II after reconstitution with various anions. The anion concentrations and the flash frequency were the same as in Figure 1. The datapoints indicate the final level of the absorbance change, read at 80 or 130 ms after 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: $S_0 \rightarrow S_1$, 1.9; $S_1 \rightarrow S_2$, 6.0, $S_2 \rightarrow S_3$, 4.2, and $Y_Z \rightarrow Y_Z^\bullet$, 2.6; offset, 0.16 (probably due to the acceptor side). The initial amount of $S_0$ was 13.1%, except for $NO_2^-$ (20.1%), and the values required for temporary inactivation of $S_3$ to fit the oscillation patterns shown were as follows: 31.5% ($Cl^-$); 31.4% ($Br^-$); 42.3% ($NO_3^-$); 33.9% ($NO_2^-$); 36.2% ($I^-$). Deactivation of the $S_3$ state to $S_2$ between flashes was 14.5% for $NO_2^-$, and it was negligible for the other anions. In the presence of $I^-$, deactivation of the $S_3$ 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 $\Delta A = 2 \times 10^{-3}$.

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 $O_2$ evolution with $NO_3^-$, $NO_2^-$, 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 $S_3$ state (*24*). This inactivation was found to be similar for $Cl^-$ and $Br^-$, slightly higher for $NO_2^-$ and $I^-$ and highest for $NO_3^-$, 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 $S_3$ state may well explain the success of the assumption of a reversible inactivation of the $S_3$ 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

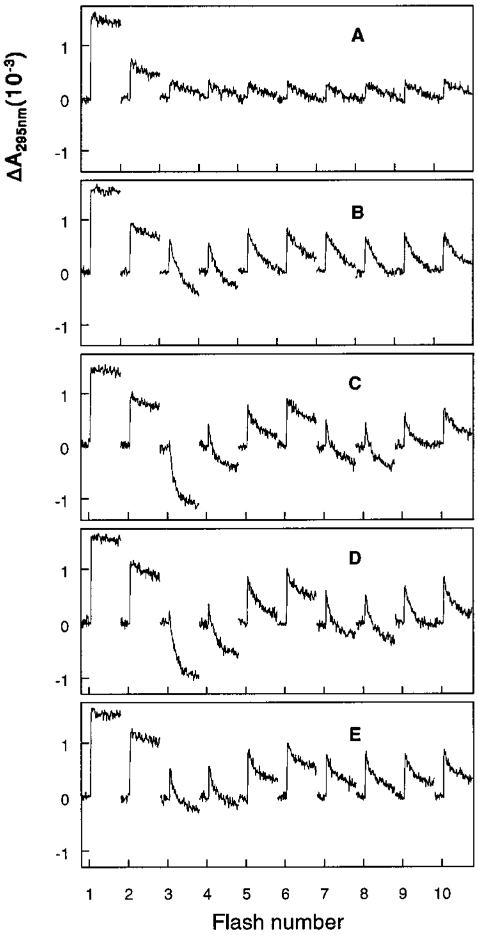

FIGURE 3: Absorbance changes at 295 nm induced by 10 saturating flashes in the following: $Cl^-$-depleted PS II (A); PS II reconstituted with 10 mM NaI (B); PS II reconstituted with 50 mM NaCl (C); PS II reconstituted with 50 mM NaCl and 10 mM NaI (D and E). The flashes were spaced at 100 ms except for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

$S_3$ state. Alternatively, the slower $S_4 \rightarrow S_0$ kinetics might cause extra misses on this transition due to charge recombination of $Q_A^-S_4$, 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 $S_4 \rightarrow S_0$ 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 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 $Y_Z^\bullet$ (*15*), from damage caused by long-lived radicals ($P_{680}+$, $Y_Z^\bullet$) or from both.

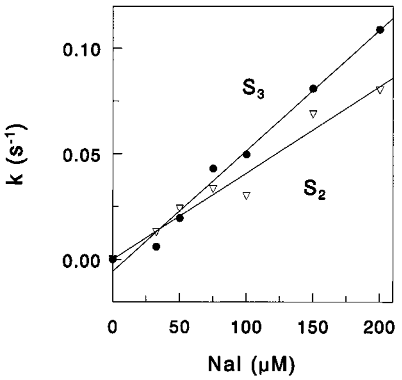

FIGURE 4: Dependence of the initial rate of reduction of the $S_2$ and $S_3$ states on the $I^-$ concentration in untreated PS II membranes at pH 6.0, in the presence of 30 mM NaCl. The samples were preilluminated with one or two flashes, and 10 s later the measurement followed (a series of 10 flashes). 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′).

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 $S_4 \rightarrow S_0$ 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 $S_2$ and $S_3$ States[a]

| | none | $Br^-$ (30 mM) | $NO_3^-$ (30 mM) | $NO_2^-$ (2.5 mM) | $I^-$ (100 $\mu$M) |
|---|---|---|---|---|---|
| $\tau(S_2 \rightarrow S_1)$ (s) | 159.5 | 87.1 | 51.1 | 121.7 | 27.5 |
| $k$ | $6.2 \times 10^{-3}\ s^{-1}$ | $0.17\ M^{-1}\ s^{-1}$ | $0.44\ M^{-1}\ s^{-1}$ | $0.77\ M^{-1}\ s^{-1}$ | $301\ M^{-1}\ s^{-1}$ |
| $\tau(S_3 \rightarrow S_2)$ (s) | 73.3 | 19.6 | 17.8 | 15.4 | 15.8 |
| $k$ | $1.36 \times 10^{-2}\ s^{-1}$ | $1.2\ M^{-1}\ s^{-1}$ | $1.4\ M^{-1}\ s^{-1}$ | $20.5\ M^{-1}\ s^{-1}$ | $496\ M^{-1}\ s^{-1}$ |

[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 $S_2$ and $S_3$ states in untreated PS II membranes, in the presence of $Cl^-$ only, are shown in column "none". The $\tau$ values are the observed decay times of $S_2$ and $S_3$ states at the given concentration of active anion. The rate constants of $S_2$ and $S_3$ reduction ($k$) were calculated by subtracting the "native" rate constants and assuming that the anion-induced rate is proportional to the anion concentration.

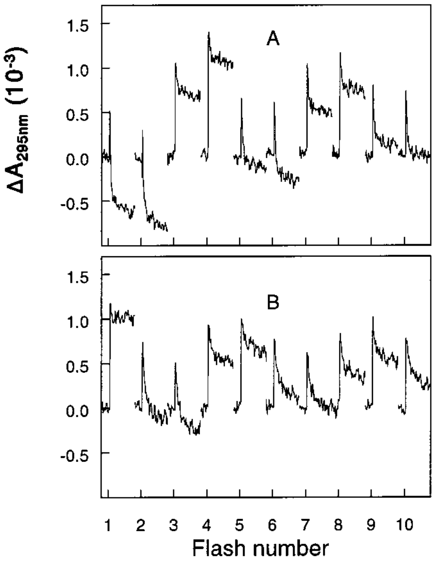

FIGURE 5: Oscillation pattern of the 295 nm absorbance changes in untreated PS II, recorded 30 s after 2 preilluminating flashes with 30 mM $Cl^-$ present. In B the preilluminating flashes were followed by injection of $NO_3^-$ to a final concentration of 30 mM.

demonstrating PS II catalyzed $I_2$ 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 $S_3$ to $S_1$ state was not seen. These observations seem to eliminate $I_2$ and $I^+$ as oxidation products of the deactivation of the higher S-states and suggest that $I^\bullet$ is the product of the deactivation reaction.

Of the other surrogate anions investigated, only $NO_2^-$ can be oxidized by $S_3$ at substantial rates, but all of these anions were found to be capable of destabilizing both $S_2$ and $S_3$ to some extent. This is illustrated for $S_3$ reduction by $NO_3^-$ in Figure 5. In these experiments, a sample was given two preflashes in the presence of $Cl^-$ to produce $S_3$. 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 $S_3$ still present in the sample at the end of the incubation period. As can be seen from the data in Figure 5, injection of $NO_3^-$ clearly destabilized the $S_3$ state. The estimated lifetime of this state with $NO_3^-$ was about 18 s, in contrast to the lifetime estimate in the presence of $Cl^-$ only (ca. 73s). The dark period between preilluminating flashes and $NO_3^-$ 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 $NO_3^-$ induced S-state reduction.

The data for all anions are summarized in Table 2. The lifetimes of $S_2$ and $S_3$ 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 $S_1$, regardless of their ability to deactivate the higher S-states. Only in the case of $NO_2^-$ a slightly enhanced $S_0$ 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, $O_2$ production in continuous light and flash-induced UV absorbance oscillations is strictly dependent on the addition of $Cl^-$ or a surrogate anion. $Cl^-$, $Br^-$, $I^-$, $NO_2^-$, and $NO_3^-$ 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 $Cl^- > Br^- > NO_3^- > NO_2^- > I^-$, 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 $S_4$ state to $S_0$, the step that accompanies the oxidation of $H_2O$ to $O_2$. The rate of this reaction decreased in the order $Cl^- > Br^- > NO_2^- > I^- >$

$NO_3^-$. 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 $S_4 \rightarrow S_0$ kinetics are unlikely to reflect differences in their binding affinities in the OEC. The anion dependence of the kinetics of the $S_4 \rightarrow S_0$ step suggests that these surrogates as well as $Cl^-$ itself may play a role in poising 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 $S_4 \rightarrow S_0$ reaction does not show any obvious correlation with ionic radius, hardness or softness of the anion, redox potential, or $pK_a$ (*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 $S_4 \rightarrow S_0$ 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 $S_4 \rightarrow S_0$ 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 $H_2O/O_2$ 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 by feasible, but it is not immediately obvious how PS II can withdraw an electron from $NO_3^-$ (the estimated $E°$ for the $NO_3^\bullet/NO_3^-$ 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.

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