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The effect of Cl^- depletion and X^- reconstitution on the oxygen-evolution rate, the yield of the multiline manganese EPR signal and EPR Signal II in the isolated Photosystem-II complex

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The role of Cl^- in photosynthetic O_2 evolution has been investigated by measurement of the steady-state O_2 rate and EPR of the electron donors responsible for the S_2 multiline signal and Signal II_s upon Cl^- depletion and substitution in Photosystem II membranes. Cl^- removal has three effects upon the donor side of Photosystem II. (1) It abolishes O_2 evolution reversibly, while decreasing the yield of the S_2 multiline signal indicative of the manganese site of the O_2-evolving complex in the S_2 oxidation state. This decrease is brought about by (2) the reversible disconnection of the manganese complex from the reaction center; and by (3) deactivation of S_1 centers having reduced primary acceptor Q_A to form S_0 centers having a reduced Signal II_s species. Reactivation of O_2 evolution by anions confirms earlier work showing a requirement for a univalent anion of optimum charge density. The observed order of reactivation is $Cl^- > Br^- \sim NO_3^- \gg OH^- \sim F^-$. Reactivation of the S_2 multiline signal follows $Cl^- \sim Br^- > NO_3^- \sim OH^- > F^-$, in near correspondence with reactivation of O_2-evolution rates. Cl^- titrations of F^--inhibited samples reveal two binding sites for Cl^- which differ in binding affinity by 11-fold. The higher-affinity site reactivates the $S_1 \rightarrow S_2$ light reaction, while the lower-affinity site reactivates the $S_3 \rightarrow S_0$ light reaction. The high-affinity site is located within the O_2-evolving complex at an undetermined site, while the lower-affinity site functions in coupling the reaction center photochemistry to the O_2-evolving complex. The results are compared with Cl^-/F^- exchange equilibria for Mn^{3+} in solution. A model for the lower S-state transitions is presented in which specific oxidation state assignments are made for some of the donors and acceptors of Photosystem II.

Introduction

Chloride is essential for photosynthetic oxygen evolution [1–5]. Recently Sinclair [6] has found that there are two sites in thylakoid membranes having different Cl^--binding affinities which influence O_2 evolution rate. Cl^- depletion has been observed to increase the yield of Signal II_f, an EPR signal arising from the oxidized form of an intermediary donor between the reaction-center

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Abbreviations: D^+, oxidized donor responsible for EPR Signal II_s; PPBQ, *p*-phenylbenzoquinone; P-680, the reaction-center chlorophyll donor; PQ, plastoquinone; PS II, Photosystem II; Z^+, oxidized donor responsible for EPR Signal II_{vf}; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; s, slow; f, fast; vf, very fast.

chlorophyll P-680 and the O_2-evolving complex [7]. This indicates a role for Cl^- in coupling these complexes. Cl^- has been postulated to function as a bridging ligand between two Mn ions of the O_2-evolving complex based upon competitive binding of Cl^- and amines to Photosystem-II particles [7]. The binding sites for amines or chloride have not been established.

The affinity for Cl^- in thylakoids from salt-tolerant plants such as mangrove decreases by 20-fold along with a parallel loss in O_2 evolution upon removing a 23 kDa peripheral protein. This reverses upon rebinding of the protein from mangrove or spinach [8]. It is possible to replace this protein with divalent ions such as Ca^{2+} [9]. These observations are consistent with a role for the 23 kDa protein in the binding of Cl^-.

The binding of Cl^- is often interpreted to influence the populations of the meta-stable oxidation states of the O_2-evolving complex, the so-called S states [10]. From flash O_2-yield experiments carried out after the readdition of Cl^- to Cl^--depleted chloroplasts, it has been concluded that the S-state distribution in dark-adapted, Cl^--depleted chloroplasts is 40% S_2, 15% S_3 and the remainder in S_0 and S_1 [11]. From delayed fluorescence experiments Muallem et al. [12] concluded that these higher S states are deactivated to S_0 and S_1 upon addition of Cl^-. This contrast with the ratio of 25 : 75 for S_0/S_1 found in normal chloroplasts.

Izawa et al. [13,14] find that subinhibitory concentrations of the well-known O_2-rate inhibitors, NH_2OH, Tris, etc., which have no effect on the O_2-evolution rates in the presence of Cl^-, do affect the rates in dark-adapted, Cl^--depleted chloroplasts when measured after readdition of Cl^-. This inhibition is observed to increase, up to 50%, with preillumination and with addition of the oxidizing agent $K_3Fe(CN)_6$. These authors attribute the inactivation observed in the Cl^--depleted chloroplasts to the attack of these inhibitors on the S_2 state, claimed to be stable even in the dark in Cl^--depleted samples. They also argue that the increase of inhibition observed in the presence of $K_3Fe(CN)_6$ could be accounted for by assuming that the S_1 state is oxidized by $K_3Fe(CN)_6$. Muallem et al. [11,12] and Izawa et al. [13,14] assume that the S_2 state is formed during chloroplast isolation steps owing to unavoidable exposure to light. No satisfactory explanation as to how the S_2 state could be stabilized against dark decay in Cl^--depleted membranes has been given. It is widely observed that S_2 relaxes to S_1 in Cl^--sufficient membranes following a flash completely within a few minutes or less in the absence of intense light [15]. Evidently, the use of other experimental techniques that enable the direct detection of the S_2 state are necessary in order to resolve questions about S-state equilibria in Cl^--depleted membranes. The S_2 multiline EPR signal directly monitors a manganese center identified with the S_2 state of the water-oxidizing enzyme [16,17]. We present in this paper the effect of Cl^- depletion, substitution with Br^-, F^- and NO_3^- and reconstitution with Cl^- on the S_2 multiline EPR signal, EPR Signal-II$_s$ and oxygen evolution rates in PS-II membranes.

Experimental

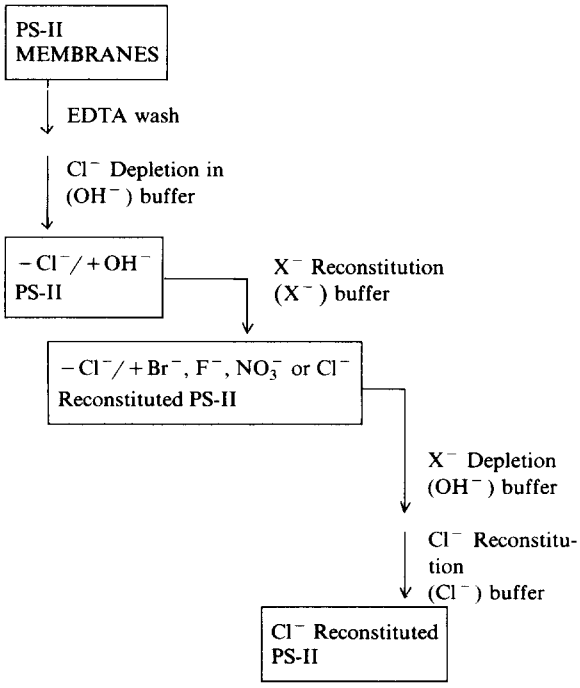
Spinach grana thylakoid membranes were obtained as described [18]. PS-II particles were obtained from grana thylakoid membranes by a slight modification of the method of Yamamoto et al. [19]. The PS-II particles obtained were highly active, showing oxygen-evolution rates of 500–600 μM $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$ at pH 6.5. The oxygen rates were assayed using a YSI model 53 oxygen monitor and a Clark-type oxygen electrode at 20°C. Electron acceptors used were 1.7 mM $K_3Fe(CN)_6$ and 1.3 mM phenyl-*p*-benzoquinone (PPBQ).

All suspension media contain 0.33 M sorbitol. In addition they contain (1) (OH^-) buffer: 10 mM Hepes (pH 7.5); (2) (NO_3^-) buffer: 10 mM Mes/4 mM $Mg(NO_3)_2$/15 mM $NaNO_3$ (pH 6.5); (3) (Br^-) buffer: 10 mM Mes/4 mM $MgBr_2$/15 mM NaBr (pH 6.5); (4) (F^-) buffer: 10 mM Mes/23 mM KF (pH 6.5); and (5) (Cl^-) buffer: 10 mM Mes/4 mM $MgCl_2$/15 mM NaCl (pH 6.5). Cl^--free media were prepared according to Ref. 3.

The basic procedures of Izawa et al. [2,13] were used for chloride-depletion experiments with some modifications. In the present work, high pH, which accelerates Cl^- removal, was deliberately avoided beause this treatment results in the release of Mn. We found pH 7.5 to be appropriate to carry out chloride-depletion experiments without loss of Mn

from the PS-II particles as evidenced by the absence of the Mn^{2+} EPR signal in the supernatants of chloride-depletion steps. The PS-II particles were washed with (OH^-) buffer containing 0.5 mM Na_2EDTA which enhances depletion of functional chloride [20], possibly by extraction of divalent metals which promote membrane stacking.

Details of the chloride-depletion experiments are given in Scheme I. The EDTA washed PS-II particles when incubated in (OH^-) buffer to give chloride-depleted membranes having 85–95% loss in O_2 rate measured in Cl^--free medium. These O_2 rates do not show a pH dependence between 5.5 and 7.5. Upon reconstitution with Cl^-, by incubation in Cl^- buffer, restoration of 95% of the O_2 rate compared to the control is obtained. Similar incubation in the NO_3^--, F^-- or Br^--containing media yielded membranes having O_2 rates of 69, 10 and 75% respectively, compared to the control. Subsequent incubation in (OH^-) buffer results in the depletion of NO_3^-, F^- and Br^- and the 95% inhibition of O_2 evolution in each. These membranes upon reconstitution with Cl^-, show 90–95% recovery of oxygen rates compared to controls. The redepletion and chloride reconstitution of Br^-, F^- and NO_3^- samples demonstrates that the anion exchange process is largely reversible, as evidence by the high O_2-rates relative to controls. Controls in all the aforementioned experiments are treated identically, except using (Cl^-) buffer of appropriate pH (6.5 Mes or 7.5 Hepes). The percentage of the O_2 rates mentioned above and in Table I are with respect to controls of that particular step. Controls for the last step in Scheme I (reconstitution of Br^-, F^- or NO_3^- samples with Cl^-) retain greater than 60% of the original O_2 rate of the starting samples. Retention of such high O_2 rates is an indication of the stability of the PS-II particles.



Scheme I. Protocol for Cl^- depletion and reconstitution of PS-II membranes with X^-. Each arrow represents an incubation step at a chlorophyll concentration of 0.5 mg/ml in the indicated buffers (see text) for 30 min, followed by centrifugation at 40000 × *g* for 20 min. All steps were conducted in the dark, and so refer to the equilibria in the S_1 state.

EPR samples are prepared using the pellets obtained in different steps of Scheme I and the appropriate controls. The pellets are suspended in the buffer last used for centrifugation and diluted with glycerol to give a final chlorophyll concentration of 6–9 mg/ml. All steps are carried out at 0–4°C. The samples are dark-adapted for 15 min, frozen to −78°C (methanol and solid CO_2 mixture) and illuminated with visible light. The light intensity at the sample is 0.9 W/cm^2. EPR spectra are obtained at 9.5 GHz on a Varian E-12 spectrometer operating with 100 kHz field modulation and fitted with an Oxford Instruments ESR-900 continuous flow cryostat.

The S_2 multiline EPR signal intensities are obtained from the peak heights and widths of the five well-defined low-field lines. The area under the five peaks was summed and normalized for chlorophyll concentration. The area under each of these transitions was calculated as (peak height) × (linewidth)^2. Linewidth changes are small and not systematic.

The O_2 rates are measured using the same samples used for obtaining EPR spectra. The appropriate suspension media are used for obtaining the rate data in the absence of glycerol and including electron acceptors. No increase in the O_2 rate of Cl^--depleted samples or F^--substituted samples is observed when 4 mM $Mg(ClO_4)_2$ is added to the suspension medium. Thus the observed O_2-rate

TABLE I

STEADY-STATE O_2 EVOLUTION RATES, YIELD OF S_2 MULTILINE EPR SIGNAL AND EPR SIGNAL II IN BOTH Cl^--DEPLETED AND Cl^--RECONSTITUTED SPINACH PS-II MEMBRANES

No exogenous electron acceptor is present except for O_2-rate measurements. The percentages are relative to the controls at the top of the columns except for the S_2 multiline intensities in the dark which are relative to the light-induced S_2 multiline yield of the control.

Anion	After Cl^- depletion				After Cl^- Reconstitution	
	$O_2\uparrow$ (%)	S_2 Multiline 200 K dark (%)	illumination (%)	Signal II dark + slow (%)	S_2 Multiline 200 K illumination (%)	$O_2\uparrow$ (%)
Cl^- (control	100	< 10	100	100	100	100
$-Cl^-/+OH^-$	5–15	< 10	20	57	96	96
$-Cl^-/+Br^-$	75	< 10	100	53	96	95
$-Cl^-/+NO_3^-$	69	< 10	20	50	85	90
$-Cl^-/+F^-$	5–15	< 10	20	51	95	95

inhibition is not due to the absence of divalent ions in the suspension medium.

Results and Discussion

If the S_2 state of the water-oxidizing enzyme is stabilized in Cl^--depleted chloroplasts, as has been claimed [13,14], then it should be possible to observe the S_2 multiline signal in dark-adapted Cl^--depleted PS-II particles. As summarized in Table I, we do not observe this signal in dark-adapted, Cl^--depleted or control PS-II particles. Because Izawa et al. [13,14] assumed that the S_2 state is formed due to low level exposure to light during dark sample preparation, we have carried out incubations in Cl^--free medium both in the dark and by deliberately exposing to room lights. In both cases no S_2 multiline EPR signal is observed in the dark-adapted samples. The same results are found for Br^--, F^-- and NO_3^--substituted membranes.

S_2 multiline EPR yield

Significant differences in the light-induced, S_2 multiline EPR signal intensities are observed between Cl^--containing and Cl^--deficient PS-II particles (Table I). In the Cl^--deficient samples without exogenous electron acceptor (PPBQ) only 20% of the multiline intensity is observed after illumination at 200 K, but increases to 96% of the control intensity when reconstituted with Cl^-. Similar results are also obtained for NO_3^--substituted membranes, where better than 85% reconstitution is found with Cl^-. For F^--substituted samples 20% multiline intensity is observed which increased to 95% upon reconstitution with Cl^-. On the other hand, 100% yield is observed in the Br^--substituted PS-II particles and this is retained upon Cl^- reconstitution. The multiline signal and O_2 rate data on the Cl^--reconstituted samples clearly indicate that the PS-II membranes remain intact after this series of treatments. Therefore, the observation of 20% S_2 multiline intensity in the Cl^--depleted, NO_3^--substituted particles and F^--substituted particles is apparently not due to irreversible denaturation of the membranes. Anion exchange is reversible.

Flash experiments

In order to test if loss of S_2 multiline signal in Cl^--depleted particles is caused by an increase in the activation energy of formation of the S_2 state, we examined the yield of the multiline signal in samples that were illuminated by a single saturating laser flash (532 nm, 20 ns width) at 0–10°C followed by quench cooling to 77 K [16]. This method of formation of multiline signal should be less susceptible to possible changes in the temperature dependence of the transfer of oxidizing equivalents from P-680$^+$ to the oxygen-evolving complex. We find that the yield of the S_2 multiline signal in Cl^--depleted and Br^--reconstituted samples is within 90% of that obtained by continuous

wave illumination at 200 K for 5 min. The rate of multiline signal formation was not investigated in Cl^--depleted samples, so that changes in kinetics of formation of the multiline signal were not investigated.

S_2 multiline increases with Hill acceptors

The PS-II electron acceptor PPBQ increases the light-induced (200 K) S_2 multiline signal intensity by 20% and 13% for Cl^--containing and Br^--substituted PS-II particles, respectively (Table II). A similar result was observed by Hansson and Andreasson in Cl^--containing chloroplasts [21]. For both Cl^--depleted and NO_3^--substituted PS-II membranes the light-induced S_2 multiline intensity increases to 60–65% in the presence of PPBQ from 20% without PPBQ. This is equal to 51–54% of the control yield with PPBQ (Table II). We interpret these results to mean that PPBQ is capable of replacing Q_B in detergent-extracted PS-II membranes that are Q_B deficient, thereby shifting the equilibrium between Q_A^- and Q_A to the oxidized form:

$$Q_A^- \xrightarrow{\text{PPBQ}} Q_A^- Q_{B'} \rightleftharpoons Q_A Q_{B'}^-$$

The fraction of centers with oxidized Q_A before illumination determines the yield of S_2 multiline signal.

Double turn-over illumination steps

In order to test if dark deactivation of S_1 centers to an S_0 level occurs upon Cl^- depletion, a double illumination experiment was devised in which two electrons could be transferred per O_2-evolving complex by continuous-wave illumination. If this occurred, it could be the cause of the decrease in yield of the S_2 multiline signal. The first illumination at 200 K causes transfer of one electron ($S_0Q_AQ_B \rightarrow S_1Q_A^-Q_B$). The reason for not being able to transfer more than one electron is that the primary acceptor is reduced and its reoxidation by $Q_A^- \rightarrow Q_B$ transfer does not occur at this temperature, as has been revealed by thermoluminescence [22]. However, by warming the samples to 250 K where this reaction takes place [22], while including PPBQ to oxidize Q_B^- (or Q_A^- directly), followed by refreezing, a subsequent illumination at 200 K might extract a second electron from the O_2-evolving complex and thereby generate S_2 from initially present S_0 states.

Experiments with the Cl^--containing samples reveal that a second illumination at 200 K subsequent to warming for a total of 15 s in a methanol bath at 300 K does not cause appreciable decay of the S_2 signal, as was previously known [15] (Fig. 1). This shows that a second oxidation of the multiline center ($S_2 \rightarrow S_3$) does not occur at 200 K even when charge separation is possible between Q_A and P-680. Similar results are observed for Br^--substituted PS-II particles. For the Cl^--depleted and NO_3^--substituted membranes this second illumination results in a 24–21% increase of multiline signal intensity from 51–54% to 75% of the control (Table II, Fig. 2). This increase can be explained by considering that double turn-over is

TABLE II

EFFECT OF PPBQ AND DOUBLE TURN-OVER ILLUMINATION ON THE S_2 MULTILINE EPR INTENSITY IN PS-II PARTICLES

Percentages are normalized relative to the S_2 multiline EPR intensity for the control sample illuminated once at 200 K without PPBQ. The percentages in parentheses refer to yields normalized to the control with PPBQ. The error limits for the signal intensity are ±5%. The second illumination at 200 K is preceded by warming for a total of 15 s in a methanol bath at 300 K. Anions are measured at pH 6.5 in all samples except $-Cl^-/OH^-$ (pH 7.5).

Anion	First illumination 200 K		Second illumination 200 K	
	without PPBQ (%)	with PPBQ (%)	with PPBQ (%)	$O_2\uparrow$ (%)
Cl^- (control)	100	120 (100)	118 (97)	100
$-Cl^-/+OH^-$	20	61 (51)	91 (76)	95
$-Cl^-/+Br^-$	100	113 (94)	109 (91)	95
$-Cl^-/+NO_3^-$	20	65 (54)	90 (75)	90

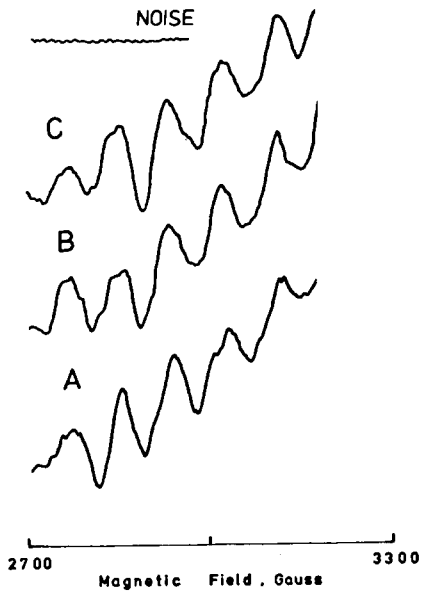


Fig. 1. Light induced (continuous wave, 200 K) EPR spectrum (9.25 GHz) part of the S_2 multiline signal in chloride containing spinach PS-II membranes in the presence of 4 mM PPBQ. (A) Single illumination (9200 K). (B) Sample A warmed for a total of 15 s in a methanol bath at 300 K and immediately refrozen. (C) Sample B reilluminated (200 K) for a second time. Temperature, 10 K; microwave power, 50 mW; modulation amplitude, 26 G; scan rate, 500 G/min; time constant, 0.3 s; illumination time, 5 min.

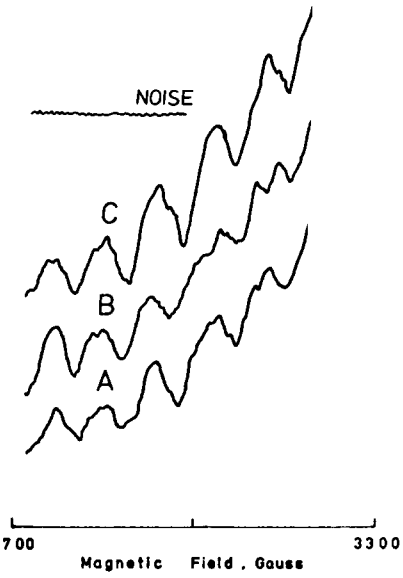


Fig. 2. Light induced (continuous wave, 200 K) EPR spectrum (9.25 GHz) of part of the S_2 multiline signal in chloride-depleted, spinach PS-II membranes in the presence of 4 mM PPBQ. See Fig. 1 for sample treatment and EPR conditions.

needed in order to generate S_2 centers from centers initially in S_0 or deactivated to an S_0 level during Cl^- extraction. This 'S_0 level' oxidation state which is reached need not be equivalent to the S_0 state which forms under continuous turn-over. It merely needs to have the same net oxidation state.

The double turn-over experiment was also performed on OH^-- and NO_3^--reconstituted samples using saturating laser pulses at room temperature followed by quench cooling. It was found that in the absence of PPBQ the yield of S_2 multiline signal after two flashes (delay, 0.55 s) was no different than the yield found after one flash. This indicates that the second flash does not create additional stable S_2 centers or destroy those formed on the first flash when there is no exogenous electron acceptor (if we exclude the possibility of off-setting effects). These results support the continuous-wave illumination results showing that the availability of oxidized acceptor controls the yield of S_2-multiline signal.

The remaining 25% loss of multiline signal (Table II) observed in Cl^--depleted and NO_3^--substituted samples compared to control seems to require another mechanism. One possible mechanism could be reversible disconnection of reaction centers from O_2-evolving complexes or from the secondary acceptor Q_B. Evidence for the first of these has been observed through the appearance of a photo-induced free radical signal in Cl^--depleted samples thought to be due to photo-oxidation of either EPR Signal-II-fast [7], or a reduced Signal-II precursor (Dismukes, G.C. and Rutherford, A.W., unpublished data). The number of spins involved relative to multiline spins is not yet known, however. Another possibility is that the multiline signal intensity is dependent on the nature of the X^- counterion. This would be likely to occur if the anion binds directly to one or more of the manganese ions forming the multiline center, thereby directly influencing the exchange interaction of the ions [23,17]. Clear evidence for direct binding is still lacking. The large multiline yield for all anions seen in Table II, once double turnovers are used to uncover the S_0 population, does not support this explanation for intensity reduction by Cl^- depletion.

It is interesting to note that the apparent equilibrium between S_0 and S_1, as measured by O_2 rate and S_2 multiline yield, is shifted towards S_0 when Cl^- is removed (presumably replaced by OH^-), or when NO_3^- or F^- are substituted for Cl^-. When Br^- replaces Cl^- this apparent equilibrium is not disturbed, even though these samples are initially depleted of Cl^- prior to reconstitution with Br^-. This suggests that although Cl^- is an apparent physiological co-factor, it is not an obligate cofactor, since other anions like Br^- function nearly as well, both in terms of O_2 rate (75%) and S_2 multiline yield (95%). Thus a common feature of Cl^- substitution by all anions is the preferential destabilization of the higher S states beyond S_2.

F^- inhibits by displacing Cl^-

Inhibition of O_2 evolution by F^- occurs even in the presence of endogenous Cl^-. We sought to learn if this inhibition is localized before or after the $S_1 \rightarrow S_2$ transition by monitoring the S_2 multiline EPR signal and O_2 rate as a function of Cl^- concentration. Fig. 3A shows the Cl^- concentration dependence for the recovery of these activities in samples originally depleted of Cl^- and containing 25 mM KF. In the absence of Cl^- there is a complete loss of the multiline signal for illumination at 200 K and of O_2 evolution. Readdition of Cl^- leads to recovery of the light-inducible multiline signal at appreciably lower concentrations than for recovery of O_2 rate, both eventually reaching a saturation limit. Each curve follows the behavior expected for equilibrium binding of Cl^- to a single type site leading to reactivation. The equilibrium constants characterizing these sites can be extracted by fitting each data set to Eqn. 1:

$$\frac{A}{A_{max}} \text{ or } \frac{V}{V_{max}} = \frac{K}{K + [F^-]/[Cl^-]} \qquad (1)$$

Here A/A_{max} refers to the fractional intensity of the S_2 multiline signal, while V/V_{max} is the fractional O_2 rate. A plot of V_{max}/V against $[Cl]^{-1}$ and A_{max}/A against $[Cl]^{-1}$ should be linear if this model is correct. K can be evaluated from the X-intercept of such a plot ($K/[F^-]$) and the measured F^- concentration. Fig. 3B presents these reciprocal plots of the data of Fig. 3A, showing that the Cl^- requirements for the S_2 multiline

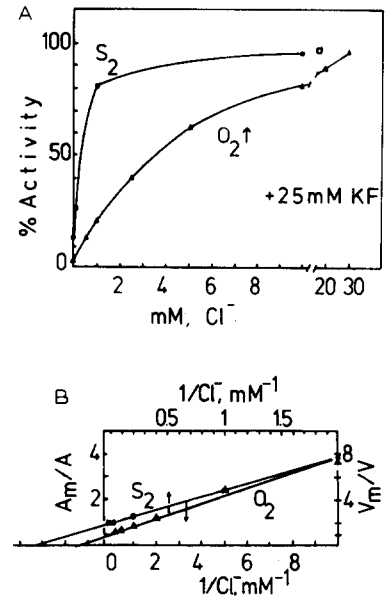


Fig. 3. (A) The dependence of recovery of O_2 evolution rate (△———△) and S_2 multiline EPR intensity (●———●) upon Cl^- concentration in PS-II membranes initially depleted of Cl^- and resuspended in buffer containing 25 mM KF. The S_2 multiline signal is generated by illumination at 200 K. Yields are normalized to 100% of control yield for samples washed in normal Cl^- buffer SM (Cl^-). (□) The S_2 multiline yield following reconstitution with Br^-. (B) Reciprocal plot of the data of part (A).

signal and for O_2 rate involve equilibrium binding to sites having different binding constants of 80 and 7, respectively. The F^- concentration used in these calculations was set equal to the total amount in solution. This is an excellent assumption at the membrane concentrations used for O_2 evolution measurements (35 μg Chl/ml), but it is not apparent that this is valid for the samples used for EPR measurements (10 mg Chl/ml). However, if non-specific anion binding were to lower the effective concentration of F^- and Cl^- in the EPR samples this should lead to a higher Cl^- requirement for reactivation of the S_2 multiline signal than for O_2 evolution. This contrasts with the 10-fold lower Cl^- requirement observed for the reactivation of the S_2 multiline signal. We can therefore deduce that non-specific anion binding does not interfere with the analysis of these data.

This is further confirmed by an estimate of the non-specific Cl^- binding using the NMR line-

$$F_a^- \cdot F_b^- \cdot E(S_1) + 2Cl^- \overset{K_a}{\rightleftharpoons} Cl_a^- \cdot F_b^- \cdot E(S_1) + F^- + Cl^- \overset{K_b}{\rightleftharpoons} Cl_a^- \cdot Cl_b^- E(S_1) + 2F^- \quad (2)$$

$$\overset{h\nu}{200\,K}\downarrow \qquad \overset{h\nu}{200\,K}\downarrow S_2 \qquad \overset{h\nu}{300\,K}\downarrow \overset{H_2O}{} O_2$$

broadening data reported in Ref. 31. These authors report an average binding constant for non-specific 'low-affinity' Cl to spinach thylakoid membranes of $K = 10\ M^{-1}$ and a site density of one per 16 Chl molecules. Using these data to approximate the extent by which the free Cl^- concentration is reduced below the total amount present, we find that this represents an insignificant reduction (less than 10^{-3}) at the EPR sample concentrations.

In summary, the Cl^- binding constants for reactivation of the S_2 multiline EPR signal at 200 K (K_a) and the rate-limiting step ($S_3 \rightarrow S_0$) in O_2 evolution (K_b) appear to be appreciably different. They refer to the halide exchange equilibria given in Eqn. 2. Cl^- binding to site (a) involves equilibration with the S_1 state of the membrane, $E(S_1)$. This site leads to recovery of the light-induced $S_1 \rightarrow S_2$ transition and has an 11-fold higher Cl^- affinity than does site (b). This latter site is involved in the reactivation of O_2 evolution, and replaces this site and shows a 7-fold higher affinity for Cl^- over F^-. The equilibrium binding constants for these sites are tabulated in Table III. Br^- was also found to reactivate the multiline signal and O_2 rate in the presence of F^- with comparable results (data not shown).

We may compare these results with the Michaelis constant of 0.5 mM reported for Cl^- dissociation in the absence of F^- from a 'high-affinity site' involved in the activation of O_2 evolution in spinach thylakoid membranes [31]. Fig. 3 yields an effective Cl^- dissociation constant for O_2 rate deactivation (site (b)) of 3.5 mM in the presence of 25 mM F^-. This decreases to about the same value as reported in Ref. 31 in the absence of F^- (data not shown), indicating that these sites have the same Cl^- affinity and so are the same.

No previous report has identified a Cl^- site essential for O_2 evolution which has a Cl^- affinity as great as that for site (a). Cl^- depletion alone, without F^- inhibition is not effective in removing Cl^- from this high-affinity site in the dark, as evidenced by the retention of the multiline signal (Table II). Although a full $[F^-]$ dependence study was not made, we can estimate the Cl^- dissociation constant at zero F^- for this high-affinity site by using the value for the low affinity site (0.5 mM) and the 11-fold ratio for K_a/K_b found at 25 mM F^-. This gives a value of 0.05 mM for the Cl^- dissociation constant for site (a) at zero F^-.

The value for K_a is measured indirectly in our EPR experiment. The fraction of centers in the dark S_1 state which is capable of being advanced to the EPR-active S_2 state at 200 K is monitored. This experiment does not allow us to discriminate between dark deactivation of S_1 and inhibition of the light-induced step $S_1 \rightarrow S_2$ as the mechanism of F^- inactivation of the S_2 multiline signal. However, illumination at 300 K where multiple turnovers are possible produces a larger percentage yield of multiline signal than at 200 K when compared to control samples illuminated at the same temperature (data not shown). This suggests that the efficiency of the $S_1 \rightarrow S_2$ transition is reduced by F^- compared to Cl^-. This indicates that F^- may interfere with the coupling between the reaction center complex and the O_2-evolving complex through dissociation of essential Cl^- from sites (a) and (b).

The data we have presented do not establish that the sites for F^- and Cl^- binding are neces-

TABLE III

EQUILIBRIUM BINDING CONSTANTS FOR Cl^- REACTIVATION OF O_2 EVOLUTION AND THE S_2 MULTILINE EPR SIGNAL IN F^--INHIBITED PS-II MEMBRANES

S_2 multiline EPR yield determined following illumination at 200 K. $[Cl^-]_{1/2} = [Cl^-]$ required for 50% reactivation at 25 mM KF.I.

	S_2 Multiline EPR ($S_1 \rightarrow S_2$) site 'a'	O_2 Rate ($S_3 \rightarrow S_0$) site 'b'	Reference
K	80	7	Eqns. 1 and 2
$[Cl^-]_{1/2}$	0.3	3.5	Fig. 3

sarily the same, although this is clearly the simplest interpretation and the one which we favor. The value for K_a in Eqn. 2 compares poorly with the value reported for Cl^- displacement of F from Mn^{3+} in aqueous solutions [33] Eqn. 3

$$MnF^{2+} + Cl^- \rightleftarrows MnCl^{2+} + F^- \quad K_{eq} = 0.028\ (2\ HClO_4) \quad (3)$$

This comparison is relevant because we know that PS-II membranes contain four manganese ions, at least two of which are essential for O_2 evolution and are organized into a binuclear unit containing two Mn^{3+} ions in the S_1 state [17]. The higher intrinsic affinity for F^- over Cl^- in Eqn. 3 is expected on the basis of the greater charge density of F^-. This is predicted to be reversed at higher pH owing to hydrolysis of bound water which forms hydroxide and oxo ligands. Stronger field ligands like F^- vs. Cl^- and OH^- vs. H_2O shift the reduction potential for $Mn^{4+} \rightarrow Mn^{3+}$ in aqueous solutions ($E_0 \approx 1.3$ V) to lower potentials, closer to the upper limit estimated for photosynthetic O_2 evolution at 0.85 V. Consequently, the available literature data on model systems are not at odds with the possible mechanism for Cl^- requirement in O_2 evolution in which this anion binds directly to Mn^{3+} and exerts thermodynamic control by maintaining the reduction potential above that required for water oxidation. Direct evidence for such binding is still lacking, however.

The Cl^- requirement for O_2 evolution rate is increased by a factor of 20–50 upon removal of a regulatory polypeptide of 23 kDa mass [8,35]. Because about the same rate is recovered at an elevated Cl^- concentration, it has been suggested that this protein acts as a 'Cl^- concentrator'. This means that the 'intrinsic' Cl^- displacement constant of the protein-depleted membranes should be about 0.35–0.14, using $K_b = 7$ for normal PS-II membranes (Table III). This 'more intrinsic' displacement constant provides a remarkably close match to the model system data of Eqn. 3, considering the conditions. This may be fortuitous. It could be helpful to study the F^-/Cl^- exchange equilibria directly with the protein-depleted PS-II membranes. A summary of the two sites for Cl^- involvement in O_2 evolution is given in the model porposed in Fig. 4.

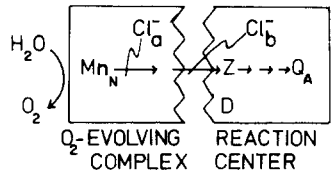


Fig. 4. This schematic drawing illustrates the location of the two sites for Cl^- binding on the donor side of PS-II which are measured in this work. Refer to the text for details.

'g = 4' EPR signal

We found no evidence for the light-induced '$g = 4.1$' EPR signal [34] in F^-inhibited samples illuminated at 200 K or 300 K. This signal is thought to arise from an electron donor to PS-II [34,40]. However, illumination of Cl^--depleted samples that were "vigorously depleted" produced this signal (data not shown, Dismukes and Rutherford, unpublished results). The signal appears strongest in disrupted preparations, leading us to speculate that it is probably associated with a structurally altered form of a PS-II electron donor, possibly Mn^{4+}.

EPR signal-II and Cl^- depletion

We sought to learn if Cl^- depletion influences the yield of other donors to PS-II. EPR Signal-II_s arises from a photo-oxidized semiquinone-like donor to PS-II which decays slowly and reversibly in the dark [29]. Underlying this photo-active signal is an identical spectrum of equal number of spins due to a photochemically inert Signal-II species. The intensity of Signal-II_s is decreased to zero upon Cl^--depletion and X^- reconstitution. As listed in Table I, there is about the same loss of Signal-II-(dark + slow) upon Cl^--depletion in the dark (43%), as upon substitution with Br^-, NO_3^- or F^- (47–50%). This loss is recoverable upon illumination at room temperature and so is attributable to Signal-II_s.

Evidence on the source of the apparent reducing equivalents responsible for the disappearance of Signal-II_s is provided in Fig. 5A. This shows the kinetics for decay of the photo-oxidized Signal-II_s species in the dark in normal Cl^--containing membranes. Upon extended dark adaptation, Signal-II_s decays completely to reveal a photochemically inactive Signal-II species having an equal number of

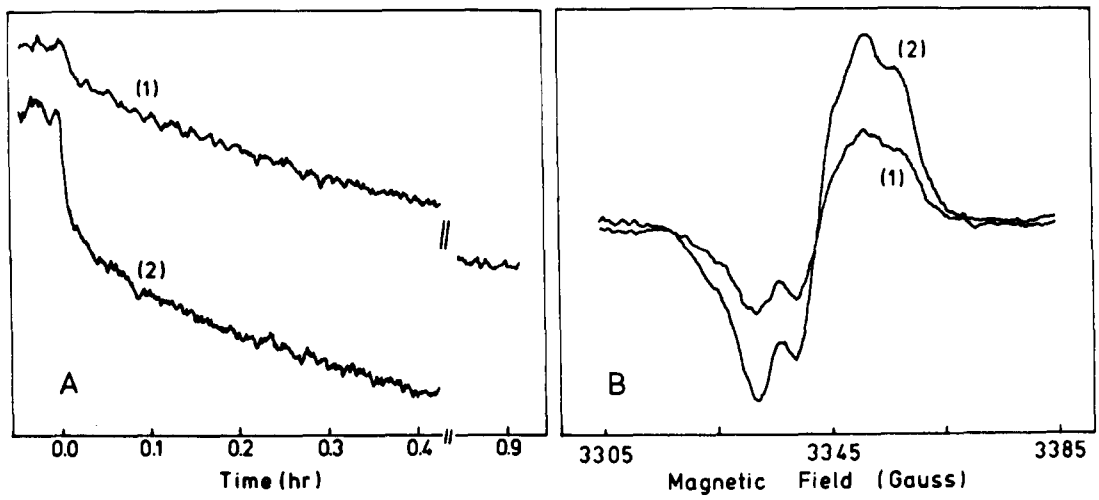


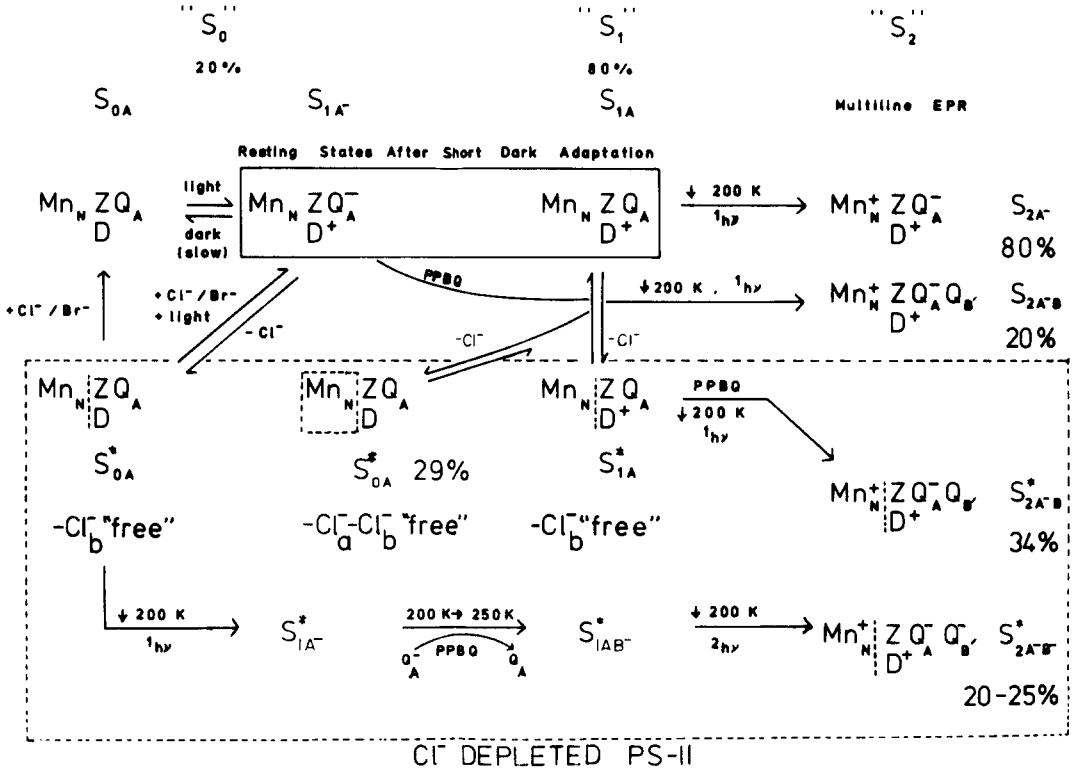
Fig. 5. (A) EPR decay kinetics for Signal-II$_s$ in PS-II particles in the dark following illumination at 300 K: (1) without externally added acceptor; and (2) with 3 mM PPBQ. In suspension medium buffer, pH 6.0. EPR, 20 mW; microwave power, 2.0G, modulation amplitude at 100 kHz, 9.5 GHz; frequency, $H = 3332$ G at the low-field first-derivative peak. (B) The amplitude of Signal-II increases 2-fold after continuous-wave illumination following a 24 h dark-adaption. Both spectra are recorded in the dark. (1) Non-illuminated; (2) after illumination.

spins (Fig. 5B). Signal-II$_s$ is abbreviated by the symbol D^+ in Scheme II. The kinetics of Signal-II$_s$ are biphasic (Fig. 5A, curve 1) with a minority component (10–20%) decaying in a half-time of 0.03 h at 300 K. A single laser flash (532 nm, 15 ns) regenerates this signal with a risetime equal to or faster than 1 s (data not shown). Addition of 3 mM PPBQ accelerates the decay of EPR Signal-II$_s$, apparently by conversion of the slower component to the faster phase of decay (Fig. 5A, curve 2). These components represent 35% (0.01 h) and 65% (0.26 h) of the total decay, respectively.

This behavior suggests that the slower decay is possibly due to the recombination of $D^+Q_A^-$ in the dark (S_1 state). The faster phase appears to correspond to recombination between D^+ and a diffusible reductant, possibly PQH_2 in centers without PPBQ. An increase in the yield of the fast phase at the expense of the slow phase upon addition of PPBQ is precisely what is expected to occur because it oxidizes Q_A^-, producing, in turn, a diffusible reductant capable of directly reducing D^+. Some reduced PPBQ is invariably present in the starting material, too. Regeneration of Signal-II$_s$ upon single flash illumination implies that oxidation of D at room temperature must occur prior to stable oxidation of the manganese center which yields the S_2 multiline signal. It is this phenomenon which appears to be responsible for the loss of the multiline EPR signal under single turn-over illumination at 200 K in Cl^--depleted membranes (Table II).

The S_0/S_1 ratio

A model is presented in Scheme II which offers an interpretation of the Cl^--depletion results. It applies to PS-II membranes which have a suppressed content of the native electron acceptor plastoquinone (PQ) as a consequence of detergent extraction. In this scheme oxidation states are assigned to the redox centers implicated in the lower S state transitions. Not all redox centers of the O_2-evolving complex or the reaction center complex are included, only those for which clear evidence exists for redox changes. The S states specify the relatively long-lived oxidation states of the entire PS-II donor/acceptor complex following flash excitations. This necessarily includes not just the components of the O_2-evolving complex, but also the reaction center components, including the quinone acceptors, Q_A and Q_B, which in their reduced form recombine with photo-oxidized donors following illumination when reoxidation by the plastoquinone pool is blocked [22]. Thus the



Scheme II. S-state equilibria: conversions between oxidation states of the PS-II reaction center and O_2-evolving complexes induced by Cl^--depletion and illumination of detergent-extracted PS-II membranes. Mn_N^+ refers to the maganese complex giving rise to the S_2 multiline EPR signal. Z and D refer to the primary and slow donors to P-680$^+$ and are equated with the plastosemiquinone-like EPR Signals-II$_{vf}$ and -II$_s$, respectively [29]. Q_A and Q_B are the primary and secondary plastoquinone electron acceptors, except that $Q_{B'}$ equals PPBQ in samples reconstituted with this alternate acceptor. Unless stated otherwise the populations refer to equilibria at 273 K, exept that illuminations are performed at 200 K. The upper line refers to normal Triton-solubilized PS-II membranes which contain Cl^-. The lower two lines enclosed in the dahsed box refer to Cl^--depleted PS-II membranes. S-state sfor Cl^--depleted samples are designated with an asterisk (*).

availability of oxidized Q_A and Q_B can control the cycling in oxidation states of the donor components. In order to include this we present an extension of the S-state nomenclature, which permits specification of the net oxidation state of both the O_2-evolving complex and of Q_A and Q_B. Thus we identify two S_1 states present in the dark in PS-II membranes in Scheme II (top line). These are labelled S_{1A} and S_{1A} to refer to states having identical net oxidation states for the donor side components (S_1), while differing on the acceptor side by having the reduced semiquinone Q_A^- vs. the oxidized Q_A, respectively. The Q_B site is unoccupied in both states. Because of the small number of PQ acceptors in equilibrium with the Q_B site in detergent-depleted PS II particles [26], and the observed stability against oxidation of Q_A [22], these two states are expected to predominate in the dark. The model in Scheme II assumes these to be the only states occupied in PS-II particles dark-adapted for short times in the absence of exogenous acceptors. 20% of the centers are presumed to occupy S_{1A^-}, which we deduce from the 20% increase in the light-induced S_2 multiline EPR signal upon addition of PPBQ (Table II). This is accounted for by oxidation of Q_A^- upon PPBQ binding to the Q_B site, followed by illumination at 200 K to yield the multiline active state S_{2A^-B}. Q_A^- must be oxidized prior to illumination in order that centers in the S_1 state can be photo-oxidized

to yield stable S_2 centers at 200 K. The remaining 80% resting population is assigned to S_{1A} centers that are photo-oxidized to the multiline active state S_{2A}.

The results suggest that centers in S_{1A} compared with the oxidized states S_{1A} and S_{1AB} have overall oxidation states (donors plus acceptors) which are formally equivalent to the traditional 'S_0' and 'S_1' states, respectively [10]. In order for this assignment to be correct it would require that centers in S_{1A} should advance to the O_2-evolving state S_4 only after four flashes, as is found for 'S_0' centers, rather than three as is found for 'S_1' type states. This possibility could arise if the S_{1A} state decays during dark adaptation to the S_{0A} state by $D^+Q_A^-$ recombination. According to the data of Fig. 5, D is the reduced form of EPR Signal-II-slow in Scheme II. Although formally equivalent to an 'S_0' type state, S_{0A} must differ from the S_0 state present under continuous turn-over conditions, because S_{0A} is not regenerated under O_2-evolving conditions where oxidation of Q_A^- occurs by Q_B and excess acceptor. The 20% S_0 population frequently observed in dark-adapted thylakoids by flash O_2 yield measurements [10] can thus be readily accounted for by the S_{1A} centers which decay by recombination to S_{0A} in the dark. Neither S_{1A} nor S_{0A} appear to form an EPR-detectable multiline signal upon illumination at 200 K.

This interpretation is consistent with the report by Vermaas, Renger and Dohnt showing that, according to flash O_2 yields, 20% of the centers in thoroughly dark-adapted chloroplasts undergo reduction with a half-life of 1–1.5 S in the S_2 and S_3 states by an intrinsic donor speculated to be the reduced form of EPR Signal II_s [27]. In support of this, Velthuys and Visser [28] found that reduction of thylakoids by DCIP and ascorbate leads to the loss of EPR Signal-II_s. Subsequent photooxidation forms S_2 from S_1, or S_3 from S_2, which are then reduced to S_1 and S_2, respectively, with reappearance of EPR Signal-II_s [28,29]. The net effect is the same as an apparent increase in the initial S_0 population if only O_2 flash yields are monitored.

Consequences of Cl^--depletion on the lower S states

The model given in the lower half of Scheme II offers an interpretation of the effects of Cl^--depletion upon the EPR signals for the S_2 multiline center and Signal-II_s (Tables I and II). For the purpose of nomenclature, in order to distinguish between Cl^--depleted and Cl^--sufficient membranes, a superscript asterisk sign is appended to the S-state symbols of the depleted samples.

A maximum 76% of the S_2 multiline EPR signal is retained under the present conditions of Cl^- depletion (Table II). With reference to Scheme II this is attributed to the sum of the S_2 yields arising from: (1) 17% of the original S_{1A} centers which are unaffected by depletion and so form S_{2A} upon illumination at 200 K; (2) 34% due to Cl^--depleted S_{1A}^* centers which are capable of advancement to an S_2 state by illumination in the presence of the exogenous acceptor PPBQ (yielding $S_{2A\ B}$). These centers appear to have been depleted of Cl^- from the (b) site only (Eqn. (2)); and (3) double illumination of centers deactivated to S_{0A}^* either from S_{0A} or by recombination of $D^+Q_A^-$ in centers originally in S_{1A} creates the balance of 21–25%. This leaves about 25% of centers which appear to lose the ability to generate the S_2 state altogether at 200 K, even with PPBQ or multiple illuminations. This is a reversible deactivation, however, since Cl^- or Br^- reconstitutes 95% of the original O_2 rate and S_2 multiline yield. This 25% reduction in multiline yield upon Cl^- depletion is accompanied by an increase in yield of a light-induced free radical signal attributed to Signal-II_s. This fraction of centers appears to have lost Cl^- from both the (a) and (b) sites, and so cannot form the S_2 multiline signal.

Cl^- depletion appears to have at least three effects upon the donor side. It inactivates O_2 evolution; it uncouples the manganese complex responsible for the multiline signal from the reaction center; and it causes S_1 centers having reduced Q_A^- to deactivate to an S_0 level. The extent to which each effect occurs is governed by the initial distribution of 'S_0' and 'S_1' centers during Cl^- depletion (a function of dark-adaptation time) and the conditions for depletion (pH, light and time).

The results from fluorescence experiments show that Cl^- depletion in the dark creates a state in which two electrons can be transferred to P-680$^+$ with blockage occurring at or beyond the $S_2 \rightarrow S_3$ transition [38,39]. This has been attributed to deactivation by recombination in the dark $S_{i+1}Q_A^-$

$\rightarrow S_i Q_A$ [38]. Both claims are in full agreement with the present work.

Conclusions

The effectiveness of anions in replacing Cl^- at site (b) is found to be $Cl^- > Br^- \sim NO_3^- \gg F^- \sim OH^-$. This is in agreement with previous studies and, as previously noted [31], indicates a requirement for a univalent anion of optimum charge density. Compared to Cl^- (ionic radius, 0.097 nm) small anions like F^- and OH^- (0.057 and 0.065 nm) inhibit O_2 evolution, while slightly larger anions like Br^- (0.112 nm) and NO_3^- are only slightly less effective than Cl^-. It is noteworthy that the higher charge density F^- ion has a 7-fold lower affinity for the (b) site than does Cl^-. This seems to contradict the expectation that F^- should bind more strongly than Cl^- if ionic bonding is responsible for the binding affinity at this labile site. Although F^- probably does have a higher affinity than Cl^- at site (b), the greater free energy of hydration of F^- over Cl^- ($\Delta G(F^-) - \Delta G(Cl^-) = -120$ kJ/mol; see Refs. 36 and 37) shifts the equilibrium towards hydrated F^-. Thus ionic bonding of a small univalent anion of optimum charge density and labile coordination is a reasonable description of the site (b) requirements. These requirements suggest that both binding and dissociation of the anion may occur during the S-state transitions, not merely static binding.

Cl^--depletion at site (a) is responsible for deactivation of S_1 centers, some of which form S_0 centers in the dark. This is reversed by addition of Cl^- or Br^-, but is unaffected by reconstitution with F^- or NO_3^-. Thus the larger ions Br^- and NO_3^-, although supporting similar O_2 rates, differ in their ability to reactivate the S_1 state, with Br^- being better. Following double-turn-over illumination to reveal all S_2 centers, the rank order of anions in terms of their yield of S_2 centers is $Cl^- \sim Br^- > NO_3^- \sim OH^- > F^-$. This again correlates with charge density of the anion.

Small anions like F^- inhibit the lower S-state transition $S_1 \rightarrow S_2$, controlled by site (a), while Cl^- depletion inhibits the higher S-state transition $S_3 \rightarrow S_0$, controlled by site (b). The degree of inhibition of the $S_1 \rightarrow S_2$ transition by F^- is reduced if illumination is carried out at 300 K instead of at 200 K and with increasing illumination intensity. This suggests that the mechanism of inhibition involves a lowering of the rate of photo-oxidation of the Mn_N center.

It is interesting to compare these results with a model proposed for the net charges which occur in the O_2-evolving complex upon the transitions $S_0 \rightarrow S_1$, $\rightarrow S_2$, $\rightarrow S_3$, $\rightarrow S_0$ [40]. Using an electrochromic shift of a carotenoid pigment in PS-II as a reporter, these authors find the net change in charge relative to the S_0 state to be $0/+1/+1/0$, which is the sum of charge changes due to photo-oxidation $(+1/+1/+1/+1)$ and proton release $(-1/0/-1/-2)$. According to this result, if Cl^- were to function so as to maintain charge neutrality it would bind on the $S_1 \rightarrow S_2$ transition and be released on the $S_3 \rightarrow S_0$ transition. This coincides nicely with the loci for F^- inhibition and Cl^--depletion, respectively. This model suggests a possible dynamic role for Cl^- in the O_2-evolving reactions; maintaining charge neutrality at a site which could involve the Mn_N site, but which remains unconfirmed.

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