Mechanism of Photoinhibition of Photosynthetic Water Oxidation by Cl^- Depletion and F^- Substitution: Oxidation of a Protein Residue†

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ABSTRACT: New evidence on the chloride requirement for photosynthetic O_2 evolution has indicated that Cl^- facilitates oxidation of the manganese cluster by the photosystem II (PSII) Tyr-Z^+ radical. Illumination above 250 K of spinach PSII centers which are inhibited in O_2 evolution by either Cl^- depletion or F^- substitution produces a new EPR signal which has magnetic characteristics similar to one recently discovered in samples inhibited by depletion of Ca^{2+} only [Boussac et al. (1989) *Biochemistry 28*, 8984; Sivaraja et al. (1989) *Biochemistry 28*, 9459]. The physiological roles of Cl^- and Ca^{2+} in water oxidation are thus linked. The characteristics include a nearly isotropic $g = 2.00 \pm 0.005$, a symmetric line shape with line width = 16 ± 2 mT, almost stoichiometric spin concentration relative to Try-D^+ = 0.6 ± 0.3 spin/PSII, very rapid spin relaxation at all temperatures measured down to 6 K, and an undetectable change in magnetic susceptibility upon formation ($<1\ \mu_B^2$). The signal appears to originate from a spin doublet (radical) in magnetic dipolar contact with a transition-metal ion, most probably a photooxidized protein residue within 10 Å of the Mn cluster (Mn-proximal radical). It is distinct from the three other protein-bound radical-type electron donors found in the PSII reaction center: Tyr-D^+, Tyr-Z^+, and C^+. This signal photoaccumulates to a stable level under continuous illumination at 270 K and decays only after illumination stops. Illumination below 250 K suppresses both photooxidation of the Mn cluster and formation of the Mn-proximal radical, with parallel formation of the C^+ radical (0.9-mT line width) in place of the usual Tyr-Z^+ signal. Either Tyr-Z^+ or the Mn cluster are candidates for oxidation of the Mn-proximal protein residue above 250 K. Single-turnover laser-flash EPR studies above 250 K show that the new signal appears after two flashes, photoaccumulates in the S_3' state, and is blocked from further turnover. Nearly fully recovery of water oxidation, low-temperature electron transfer (Mn → Tyr-Z^+), and loss of the Mn-proximal EPR signal occur upon Cl^- reconstitution. These observations support earlier studies suggesting that photooxidation of a species other than Mn may occur during normal photochemistry in the native enzyme. F^--substituted PSII centers exhibit a large increase in magnetic susceptibility for the $S_1' \rightarrow S_2'$ state transition that is indistinguishable from Cl^--reconstituted samples, indicative of an equivalent decrease in magnetic coupling between the Mn ions of the cluster for both halides. Therefore, the $S_1 \rightarrow S_2$ oxidation step in F^--substituted centers cannot occur at a magnetically isolated Mn(III) monomer site remote from the Mn cluster, as had been suggested earlier by others on the basis of formation of an EPR signal at $g = 4.1$. The large increase in magnetic susceptibility is consistent with simultaneous Mn oxidation and magnetic uncoupling of a tri- or tetranuclear Mn cluster on the $S_1 \rightarrow S_2$ transition [Sivaraja et al. (1989) *J. Am. Chem. Soc. 111*, 3221].

Photosynthetic O_2 evolution from water takes place at a manganese cluster which binds at the interface of the photosystem II reaction center protein complex and the so-called manganese-stabilizing protein (Miyao & Murata, 1985). Both calcium and chloride are essential physiological cofactors for water oxidation and bind to specific sites within this metalloprotein complex.

While a great deal is known about the phenomenology of the chloride requirement, the mechanism by which Cl^- exerts its influence remains a matter of speculation. Chloride is not required for the primary photochemistry in the PSII reaction center but is involved in the water-oxidizing apparatus (Govindjee et al., 1983). It appears to not undergo direct oxidation itself, since other anions can substitute for it, but does influence the structure and redox properties of the manganese cluster where water is oxidized. Other anions including Br^- and to a lesser extent NO_3^- can functionally replace Cl^- at the site which specifically affects the O_2-evolving step, the so-called $S_3 \rightarrow S_0$ transition (Kelley & Izawa, 1978; Homann, 1987; Govindjee, 1989b). The critical requirement appears to be the charge density (Critchley et al., 1982; Govindjee & Homann, 1989a), with anions of smaller ionic radius being inhibitory (F^- and OH^-) and larger ones being ineffective (ClO_4^-, SO_4^{2-}, and HPO_4^{2-}). The reversible inhibition by smaller anions suggests that the ionic binding strength is the determining factor.

There are several types of chloride binding sites in intact thylakoid membranes which have been distinguished on the basis of their binding affinities and their influence on the S-state transitions. Part of the complexity of the chloride requirement originates in the heterogeneity of binding sites and their dependence on the extrinsic polypeptides of the water-oxidizing complex [see reviews by Miyao and Murata (1985), Govindjee and Homann (1989a), and Homann (1988)]. Baianu et al. (1984) and Govindjee et al. (1983) estimated that a large pool of 16 and 25 Cl^- PSU (1 PSU = 400 Chl in thylakoids) is involved in stimulating O_2 evolution in intact thylakoid membranes from spinach and halophytes,

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respectively. On the basis of $^{35}Cl^-$ NMR line broadening investigations showing maxima at approximate concentrations of 0.25, 0.85, 2–3, and 6–7 mM Cl^-, Coleman et al. (1987a) proposed that as many as four binding sites exist. Because these were lost by NaCl washing to remove the extrinsic 18- and 24-kDa proteins, they were assigned a regulatory rather than catalytic function (Coleman et al., 1987b). The more loosely bound of these exerts an indirect influence on O_2 evolution, involving nonspecific sites which are suspected to correlate with changes in the stacking of thylakoids and energy transfer between chlorophyll. Consequently, these sites may influence O_2 rates by controlling how efficiently light is utilized.

A second pool of "intermediate" binding affinity directly influences the kinetics of the O_2-evolving step, the $S_3 \rightarrow S_0$ transition (Sinclair, 1984). This site, termed the "b" site, has a dissociation constant of 0.35–0.6 mM (Baianu et al., 1984; Sandusky & Yocum, 1984). It appears to correlate with the binding of 4–5 Cl^-PSU on the basis of studies using the radioisotope $^{36}Cl^-$ (Theg & Homann, 1982). This pool may also correlate with about five titratable positive charges on the PSII complex which can be neutralized with the competitive anion OH^- (Critchley, 1985).

The third site, denoted the "tight" or "a" binding site, is characterized by 11-fold stronger Cl^- binding than the intermediate site (Damoder et al., 1986). Cl^- binding to this site occurs in the dark in the S_1 oxidation state. Photooxidation of Cl^--depleted PSII samples yields an abnormally stable S_2' oxidation state (Izawa, 1983). This is characterized by loss of the normal protein conformation which exhibits the S_2-state manganese multiline EPR signal and its replacement to varying extents by an unstructured $g = 4.1$ signal (Casey & Sauer, 1984; Ono et al., 1987a), and by thermodynamic stabilization, as seen by an increase in the temperature for stimulation of thermoluminescence by charge recombination (Ono et al., 1986a, 1987; Homann et al., 1986; Vass et al., 1987; Rosza & Demeter, 1987). The Cl^- stoichiometry has not been measured for the a site. It has been difficult to pin down the location of this binding site. Spectroscopic methods for looking at the Mn cluster such as EPR of the S_2-state multiline signal and EXAFS have failed to see changes upon substitution of Cl^- by Br^- (Yachandra et al., 1986a,b). This has been interpreted in favor of indirect conformational coupling to the Mn cluster via a remote Cl^- site, rather than direct binding to Mn. However, both methods could miss detection of a small number of exchangeable halide ligands coordinated directly to the Mn cluster, for which a total of 24 first-shell donor ligands are anticipated for the 4 Mn ions.

Two issues which remain unclear regarding Cl^- depletion are the number of electrons which can be extracted before inhibition is expressed, and whether the a and b sites represent a single type site differing only in its affinity as measured in the S_1 and S_3 oxidation states, respectively. If there really is a single type of specific catalytic site which accounts for the different a and b binding affinities, then it should be expected that Cl^- depletion would block turnover at a single S-state transition. As summarized, the literature reports are ambiguous on this issue.

Direct evidence from ^{35}Cl NMR has shown that some Cl^- binds dynamically, with binding from solution occurring in the S_2 and S_3 states and release on the $S_3 \rightarrow S_0$ transition (Preston & Pace, 1985). However, this may involve loosely bound chloride related to charge neutralization rather than the tightly bound Cl^-. Under physiological salt concentrations found in the lumen of thylakoids, both the a and the b chloride sites should be fully occupied, thus precluding a regulatory role at these sites for Cl^- on electron transport. Further evidence against a dynamic binding/rebinding of Cl^--linked to O_2 evolution has been provided by Izawa et al. (1969) and by Theg et al. (1984), who found that the effect of Cl^- deficiency on the rate of O_2 evolution is independent of the light intensity and hence S state.

There are potentially three physiological electron donors to the reaction center chlorophyll, P_{680}^+, in addition to the usually oxidized tyrosine-D^+. These are the Mn cluster, cytochrome *b*-559, and tyrosine-Z. On the basis of flash-induced fluorescence yield experiments (Theg et al., 1984; Itoh et al., 1984) and thermoluminescence (Homann et al., 1986), it has been found that only two electrons can be transferred to P_{680}^+ in Cl^--depleted membranes, yielding as the terminal state either S_3 or S_2Tyr-Z^+. This contrasts with more recent reports by Vass et al. (1987) and by Rosza and Demeter (1987) in which thermoluminescence revealed a block after a single turnover at the $S_2 \rightarrow S_3$ transition. This agreed with EPR studies (Ono et al., 1986, 1987). These discrepancies are presumed to originate in the complexity of the effects of Cl^- depletion. For example, a variable extent of reduction of tyrosine-D^+ occurs under the mildly alkaline conditions which deplete Cl^- and so adds another electron donor (Velthuys & Visser, 1975; Damoder et al., 1986).

Two opposing views of the consequences of substitution of Cl^- by F^- have been based upon the observed conversion of the $g = 2$ multiline EPR signal for the Mn cluster associated with the normal S_2 state to a structureless $g = 4.1$ form. One proposal attributes these signals to interconversion between two coordinatively independent Mn centers, a binuclear Mn_2(III,IV) site and an isolated Mn(IV) site, respectively, which are in redox equilibrium (Aasa et al., 1987, 1988; Hansson et al., 1987). An alternative proposal is that both the multiline and $g = 4.1$ signals arise from a single tetramanganese cluster in two different conformational states (dePaula et al., 1986; Zimmermann & Rutherford, 1986).

We have obtained new experimental data on Cl^--depleted and F^--inhibited PSII membranes and core complexes by using single-turnover laser flashes with detection of magnetic susceptibility and EPR of tyrosines-D^+ and -Z^+, the S_2-state signals for manganese, and a new signal originating from a Mn-proximal radical. This has enabled us to explore the issues described above, and has provided further support for the tri- or tetranuclear nature of the Mn cluster responsible for water oxidation.

Materials and Methods

Spinach PSII membranes containing ca. 200–250 Chl/PSII were prepared by Triton extraction (Berthold et al., 1981). O_2-evolving reaction center core complexes containing ca. 70 Chl/PSII were obtained from PSII membranes by extraction with octyl β-D-glucopyranoside (OGP) as outlined by Ghanothakis et al. (1985) with modifications as given by Sivaraja and Dismukes (1988). Typical O_2 evolution rates were 350 and 600 mmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, respectively.

Both the depletion of chloride and the exchange with fluoride were performed as outlined by Damoder et al. (1986). Briefly, chloride-depleted membranes were prepared by washing twice with Cl^--free buffer at pH 7.5 [0.4 M sucrose (ultraclean, ICN)/50 mM HEPES, pH 7.5]. Samples were then stored at 210 K in sample buffer at either pH 6 (50 mM MES) or pH 7.5, or substituted with fluoride buffer (0.4 M sucrose, 50 mM MES, pH 6–6.5, and 25 mM KF) prior to storage. Cl^--depleted samples also contained 15 mM $CaSO_4$ and 0.5 mM Na_2EDTA added prior to measurements. Care

must be taken in the use of calcium ions in the fluoride buffer due to the low solubility of CaF_2 (K_{sp} = 3.3 × 10^{-11}, 18 °C, 0.0016 g/100 cm^3; CRC Handbook of Chemistry and Physics). Cl^--free buffers were assayed by light scattering with $AgNO_3$ for residual amounts of chloride. Oxygen evolution rates were measured with a Clark electrode in the presence of 1 mM DCBQ as electron acceptor.

Reconstitution of chloride for O_2 measurements were performed either by washing F^--containing membranes or core complexes with calcium-free chloride buffer before using the calcium-containing buffer for O_2 evolution measurements or, in the case of chloride-depleted samples, by directly resuspending the samples in O_2 buffer [O_2 buffer: 0.4 M sucrose, 50 mM MES (pH 6), 15 mM NaCl, and 15 mM $CaCl_2$].

X-Band EPR measurements were performed as previously described (Sivaraja, 1989). PSII samples for EPR and magnetic susceptibility measurements contained 1–2 mM DCBQ. Flash-induced changes in magnetic susceptibility were measured with a superconducting SQUID magnetometer built by one of us (J.S.P.), based on principles previously demonstrated (Philo & Fairbank, 1977). Typically a train of six laser flashes at 570 nm and 500-ns duration (Candella SLL 625) and energy 20–50 mJ/pulse was delivered to the sample through an 8-ft-long 2-mm fiber optic cable every 2 s. The chlorophyll concentration of samples was kept low (0.7–1.5 mg/mL) and the laser intensity sufficiently high to ensure light saturation. Samples were dark-adapted for at least 15 min prior to measurement. A silvered quartz NMR tube of 7.5-mm diameter supported the sample. Data were recorded with a 300-ms time constant filter to improve the signal-to-noise ratio.

Results

EPR. EPR measurements of PSII membranes in which F^- has been substituted for Cl^- confirmed earlier reports that formation of the multiline signal associated with the normal S_2 state by illumination at 195 K is absent or greatly diminished while the g = 4.1 signal associated with the Cl^--depleted/F^--substituted S_2 state was usually weakly enhanced by a factor of 1.5–2. We have extended these measurements to O_2-evolving core complexes and also observe loss of the multiline signal. Both EPR signals originate from manganese. This comparison is given in Figure 1A,B, showing F^--substituted and Cl^--reconstituted PSII membranes, respectively. The electron acceptor DCBQ is present in these samples to ensure maximum photooxidation of the reaction center, via oxidation of Q_A^-. The F^--substituted samples had greatly lowered O_2 evolution rates of 10–20% of the control rate, while Cl^--reconstituted samples had typical rates 70–80% of the control activity.

When the F^--substituted samples are illuminated at 270 K and frozen under illumination, so that thermal activation barriers to multiple electron-transfer reactions are overcome in the normal Cl^--sufficient PSII complex, the S_2-state multiline signal remains absent while the small S_2-state g = 4.1 signal decreases further by more than 50%. Illumination also results in formation of a new EPR signal at g = 2.00 ± 0.005 centered around the signal due to Tyr-D^+, but having a broader line width, as shown in Figure 1C. Compare this to an identical sample in Figure 1D which is restricted to a single turnover by inclusion of DCMU and does not exhibit this new signal. The new signal can best be seen as the difference spectrum (Figure 1E) of sample 1C taken against an identically illuminated Cl^--reconstituted sample. The new signal does not form under the latter conditions, while Tyr-D^+ is fully photooxidized and hence subtracts in the difference spectrum.

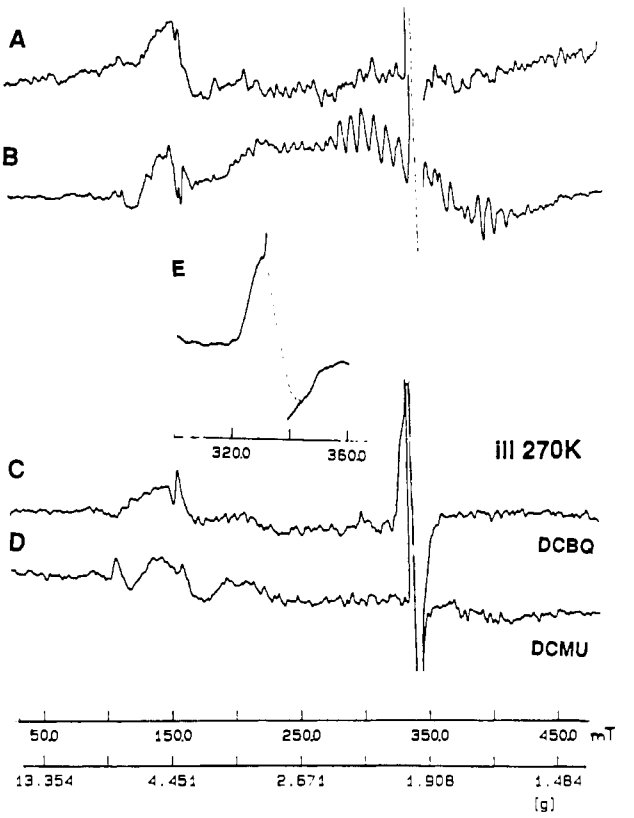

FIGURE 1: Light-minus-dark EPR difference spectra of PSII membranes that were Cl^- depleted and F^- substituted, then illuminated at (A) 195 K for 2 min or (C) 270 K for 60 s, and frozen immediately. (D) Same as (C) except this sample contains 50 μM DCMU; (B) Cl^--depleted PSII membranes that were reconstituted with Cl^- and illuminated at 195 K for 2 min. All samples except (D) contain 1–2 mM DCBQ. (E) is an expansion of the g = 2.00 region of sample C but presented as a light-minus-light difference spectrum with a control sample (Cl^- reconstituted) of the same Chl concentration (3 mg/mL). The dotted line extrapolates the spectrum through the region of interference with the Try-D^+ signal. The spectrometer conditions were the following: T = 8 K; microwave frequency, 9.345 GHz; microwave power, 20 mW; 2.0-mT pp modulation amplitude at 100 kHz.

It is not possible to completely subtract the free radical signals. The broad new signal possesses an unresolved line width of 16 ± 2 mT. If the sample is warmed again above 250 K in the dark for 10 min, the new signal disappears reversibly. This signal has not been previously reported in F^--substituted samples. It appears as though it was overlooked in part because the spectrum overlaps the spectrum of Tyr-D^+, and the conditions needed for optimum generation and detection were not evident.

If chloride-depleted PSII membranes or core complexes are used instead, the yield of this new signal is diminished to 20–60% of the maximum value observed for F^--substituted samples, depending on the extent of Cl^- extraction, as infered by the yield of O_2 evolution and the S_2 multiline signal. In the absence of a suitable anion such as F^-, the Cl^- depletion conditions were usually insufficient to remove all of the tightly bound Cl^-. Cl^--depleted samples were more unstable toward Mn release, heat, and light sensitivity than were F^--substituted samples. The yield of the multiline signal in Cl^--depleted PSII membranes was typically 20–60% of the control yield vs 5–15% in F^--substituted samples. The O_2 evolution rates followed in parallel. In general, lower yields of the 16-mT broad signal along with higher O_2 rates and multiline intensities were measured whenever the Cl^--depleted samples were stored at pH 6 where the chloride binding affinity is stronger (Theg & Homann, 1982; Izawa et al., 1983; Homann, 1988).

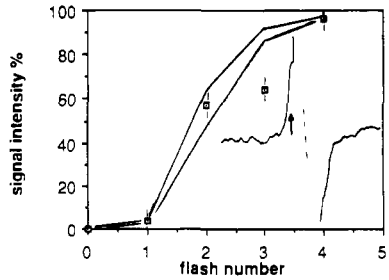

FIGURE 2: Dependence of the light-minus-dark EPR difference amplitude for the 16-mT broad $g = 2.00$ EPR signal in F^--substituted PSII membranes on the number of saturating laser flashes. The solid lines are given for a Kok model assuming a block at the S_3 state, 10% misses, 5% double hits, and initial populations of (A) 75% S_1 + 25% S_0 and (B) same as (A) plus 25% Tyr-D reduced. Instrumental conditions as in Figure 1, except 10-mW micro power. See insert for field position.

The amplitude of the 16-mT broad signal in F^--substituted samples was the same with 20, 40, or 60 mM KF in the sample buffer, indicating saturation of the F^--site, also in agreement with our prior work (Damoder et al., 1986). The intensity of this new signal reached a maximum under continuous illumination in less than 30 s and did not diminish during 90-s illumination prior to freezing. Therefore, the signal arises from an oxidation state which can be photoaccumulated and is stable under continuous illumination.

Evidence that a single turnover occurs in F^--substituted samples containing DCMU can be seen in Figure 1D from the formation of some S_2-state EPR signal at $g = 4.1$ and the appearance of an EPR signal at $g = 1.82$ due to Q_A^-Fe. The latter signal can be seen best by measurements at lower temperature and higher microwave power than used in Figure 1D. The 16-mT broad signal is also absent if no additional acceptor such as DCBQ is added (not shown). From this, we conclude that the species responsible for the 16-mT broad signal arises in a higher oxidation state than S_2.

S-State Identity. In order to ascertain which S state gives rise to the symmetric signal, a flash experiment was performed in which a F^--substituted sample was exposed to a train of laser flashes, each capable of inducing a single turnover of the PSII complex. Flashes from a Q-switched YAG laser were employed at 5-s intervals, each 15 ns in duration at 532-nm wavelength and of sufficient intensity to saturate all centers ([Chl] = 1.8mg/mL). The data, given in Figure 2, show that the broad $g = 2.00$ signal first appears after two flashes with up to 50–60% of the maximum yield. The yield after four flashes is within 95% of that formed by continuous illumination.

Spin Concentration. Spin quantitation was difficult owing to the overlapping spectra from Tyr-D^+ and C^+ and the 16-mT broad signal. This was further aggravated by the instability to decay of Tyr-D^+ in Cl^--depleted samples which thus contributes to all light minus dark difference spectra. To overcome this, we used the dark-stable Tyr-D^+ EPR signal in Cl^--reconstituted samples as an internal standard for estimation of the 16-mT broad signal by two methods, electronic double integration of difference spectra and graphical integration. The electronic double integration method was used to estimate the area of the difference spectrum of F^--substituted samples minus an equally illuminated Cl^--reconstituted sample. This enables subtraction of the overlapping Tyr-D^+ spectrum but requires matched samples. A graphical method was used to estimate relative areas of Tyr-D^+ and the broad $g = 2.00$ signals in the same sample by using their measured peak heights and line widths for unsaturated signals, plus the approximation applicable to simple Gaussian curves: area = $(\Delta H_{pp})^2$/(peak height). A Gaussian line width is not observed for Tyr-D^+, but this approximation introduces a small error compared to other sources and works remarkably well. These methods yielded 0.6 ± 0.3 spin/PSII, assuming one Tyr-D^+ per PSII (Debus et al., 1988). The normalization of one Tyr-D^+/PSII in Cl^--reconstituted samples was made by comparison to the yield of Tyr-D^+ in PSII membranes which had not been subjected to the Cl^- depletion and reconstitution procedures.

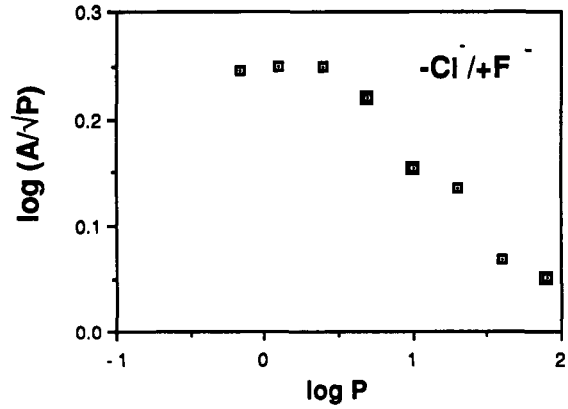

FIGURE 3: Microwave power saturation of the broad $g = 2.00$ in F^--substituted PSII membranes at 7.8 K. See Figure 1 for instrument conditions.

Spin Relaxation. From the microwave power dependence of this signal at 7.8 K, a plot of the peak height divided by $P^{1/2}$ against log P (Figure 3) revealed a flat power-independent behavior up to a threshold of $P_0 = 2.4$ mW. A linear decrease occurs above this power owing to saturation. The power necessary for this ratio to decrease to half its maximum value (P^*) was found to be ca. 300 mW by extrapolation of the data above the threshold for saturation. This may be compared to $P_0 = 1.9$ mW and $P^* > 125$ mW at 7.9 K for the analogous signal observed in calcium-depleted (citrate pH = 3, treated) PSII samples (Sivaraja et al., 1989b; Tso et al., submitted for publication).

For comparison with the literature, we need to introduce another characteristic saturation parameter. The plotting format used to represent the data in Figure 3 enables convenient graphical analysis based upon the theoretical formula (eq 1) (Yim et al., 1982). Here I = peak height, K is a

$$I = \frac{K\sqrt{P}}{(1 + P/P_{1/2})^{0.5b}} \quad (1)$$

constant, b is a parameter which varies between 1 and 4 for different line shapes but which is constant for a given line shape, and $P_{1/2}$ which is called the half-saturation power and must be equal to P^* when $b = 2$. Boussac et al. (1989) used eq 1 to analyze their experimental data and obtained $P_{1/2} = 0.8$ mW at 5.9 K for the analogous $g = 2.00$ signal induced in PSII membranes depleted of calcium by washing with NaCl/EGTA. In comparison, the PSII tyrosine radical D^+ saturates far more easily at $P^* = 0.1 \pm 0.01$ mW at 2–20 K (Isogai et al., 1988), while the S_2 multiline signal for the Mn cluster in Cl^--sufficient membranes exhibits a range of weak saturation from $P_{1/2} = 3.7$ mW to above 156 mW at 6 K, depending on the conditions of formation (dePaula & Brudvig, 1985). This comparison indicates that efficient spin relaxation of the broad $g = 2.00$ EPR signal occurs which is more typical of transition-metal ions or clusters than of a magnetically isolated free radical.

Tyrosines-D^+ and -Z^+. Figure 4 presents EPR spectra in the $g = 2$ region recorded at low microwave power to favor

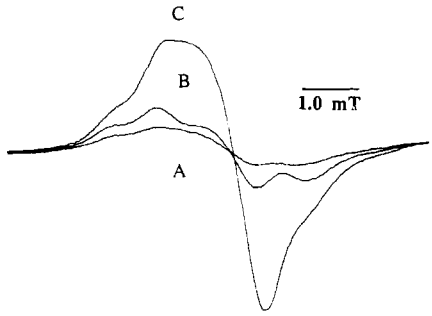

FIGURE 4: EPR signal changes for Tyr-D$^+$ and Tyr-Z$^+$ in F$^-$-reconstituted PSII membranes. (A) Dark prior to any illustration; (B) dark after preillumination at 270 K before freezing; (C) dark after further illumination at 195 K of sample B. Samples were measured at 8 K. EPR intensities were computer-integrated, yielding a ratio of reas for curve C/curve B = 2.21. EPR conditions: 100-kHz, *f*, modulation; 0.2-mT modulation amplitude; 10 mT/22-s scan width/time; 20-μW power; 2 mM DCBQ.

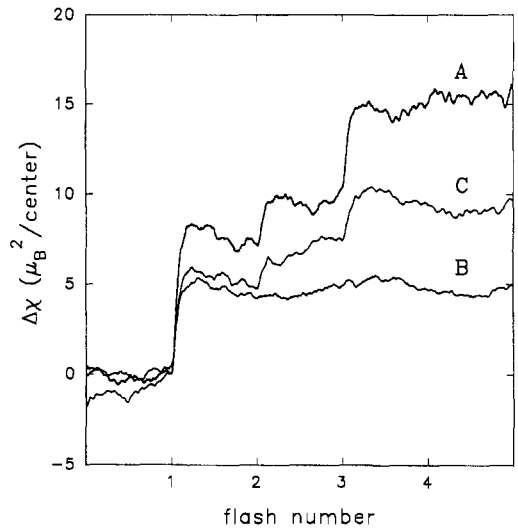

FIGURE 5: Flash-induced changes in magnetic susceptibility in spinach PSII core complexes: (A) untreated control (core stock); (B) F$^-$-substituted; (C) Cl$^-$ restored after removal of F$^-$ from (B). The data are an average of two samples each receiving a single train of six laser flashes. Chlorophyll concentration, 0.6 mg/mL, pH 6.1, 2 mM DCBQ, T = 273 K.

detection of free radicals. The normal signal II spectral intensity for Tyr-D$^+$ is decreased in Cl$^-$-depleted/F$^-$-substituted samples as shown by curve A. This signal is unstable on standing even at 77 K. Brief preillumination at 270 K prior to freezing in the dark restored the Tyr-D$^+$ signal to the control level of the Cl$^-$-sufficient sample (curve B). Subsequent illumination at 195 K results in a further increase of the EPR intensity, but with a different asymmetric line shape and line width, ΔH_{pp} = 0.91 mT (curve C). The additional area of the light-induced species is equal to 1.2 spins when referenced to the Tyr-D$^+$ signal (curve B). This additional increase did not include more than 10% contribution from Tyr-D$^+$. The control sample exhibited a much smaller light-induced increase equal to 0.4 spin (not shown). The latter signal is even smaller in samples that are more active in O_2 evolution. This behavior shows that, unlike the control sample where normal electron donation from the Mn cluster serves to re-reduce Tyr-Z$^+$ even at 195 K, Cl$^-$ depletion/F$^-$ substitution causes this pathway to be blocked. Illumination thus oxidizes about 0.8 free radical donor per PSII in these samples. The narrower line shape observed for this donor, which we shall call C$^+$, suggests two possibilities (Tso et al., 1990a). Either a different environment exists for Tyr-Z$^+$, or the species which is oxidized is not Tyr-Z. The former possibility would also require a greatly reduced rate of recombination with Q_A^-. If this sample is warmed to 273 K in the dark, the 0.91-mT-wide signal disappears reversibly. The species responsible for this spectrum is not the same species responsible for the 16-mT broad signal. The latter signal occurs in place of the C$^+$ spectrum when illumination is done above 250 K. An activation barrier thus exists for formation of the 16-mT broad signal. The C$^+$ spectrum is analogous to that which forms in PSII centers in which all other available electron donors have been photooxidized through multiple turnovers (Malkin & Bearden, 1973; Nugent et al., 1981; dePaula et al., 1985). These authors have suggested that the C$^+$ radical may be formed upon oxidation of a chlorophyll or carotenoid molecule.

Magnetic Susceptibility. Previously we have shown that magnetic susceptibility changes associated with the S-state transitions can be used to characterize the magnetic interaction between Mn ions within the water-oxidiing complex in PSII (Sivaraja et al., 1989a). Because this is a nonresonance technique, all S-state transitions contribute, and each can be assigned only starting with a synchronized population in the dark (S_1 state). Figure 5 presents the data for such an experiment measured at 273 K using PSII core complexes that have been dark-adapted for 15–20 min following preillumination. As previously reported, the control sample exhibits a large first-flash signal (mostly $S_1 \rightarrow S_2$) corresponding to $\Delta\chi = 9\ \mu_B^2$/PSII. This value is normalized to the total PSII population, including dead centers, on the assumption of 70 Chl/PSII. It thus represents a lower limit to the true value. It also varies with the quality of the preparation. The second flash, corresponding predominantly to the $S_2 \rightarrow S_3$ transition, yields a much smaller increase, while the third flash increases again and the fourth flash gives little change. The increase observed on the third flash in the control can be partly suppressed by adding glucose/glucose oxidase/catalase to scavenge O_2 formed on the $S_3 \rightarrow S_0$ transition (Sivaraja et al., 1989a). Figure 5 also compares this with a F$^-$-substituted core complex. The susceptibility increases on the first flash by 6 μ_B^2/PSII, and little further change occurs on the second, third, or fourth flashes. If F$^-$ is removed and Cl$^-$ is restored by dilution, centrifugation, and resuspension, this same sample exhibits the third pattern shown in Figure 5. Here we see about 70% recovery of the original signal intensities on all flashes, including those seen on the second and third flashes in the control. This recovery matches the extent of recovery of O_2 evolution in the same sample. The decrease in signal compared to the untreated control is typical of the loss arising from inactivation of the sample due to the treatment. These data show that the $S_1' \rightarrow S_2'$ transition in F$^-$-substituted (and Cl$^-$-depleted) samples has a large increase in magnetic susceptibility which is indistinguishable from that seen in the Cl$^-$-reconstituted control sample. The absence of detectable changes on flashes 2, 3, and 4 indicates blockage of *normal* turnover.

Discussion

Loss of Mn → Tyr-Z$^+$ below 250 K. The EPR data in Figure 1A,B for F$^-$-substituted samples, showing that loss of the multiline signal and hence loss of normal S_2-state formation upon illumination at 195 K, agree with earlier work (Casey & Sauer, 1984), and also fit well with the data in Figure 4, showing that stable photooxidation to form one C$^+$ radical occurs instead. Thus, F$^-$ substitution causes a block at 195 K in photooxidation of the Mn cluster such that either a modified Tyr-Z$^+$ becomes stably oxidized or it oxidizes an unidentified species to form a new radical, the C$^+$ species. The

observed EPR signal with a g value equivalent to that observed for signal IIs ($Tyr\text{-}D^+$) and the symmetric line width of 0.91 mT offer little clue as to its molecular identity. As discussed previously, candidates that have been suggested include chlorophyll and carotenoid radicals. A third possibility that it may be due to the special photoactive chlorophyll, P_{680}^+, is not compatible, since this would not be stable owing to back reaction with Q_A^-.

Along with the former two possibilities, we suggest a third possible origin in which the C^+ spectrum is attributed to a structural modification of the normal $Tyr\text{-}Z^+$ spectrum which reduces the hyperfine field from about 2.1 to 0.91 mT. In order for reduction in the hyperfine field to occur to this extent, the spin density, which predominantly occurs at the 1,3,5-ring carbons within the phenolic radical (Barry et al., 1990), would have to decrease, and the orientation of the two methylene protons at position 1 relative to the ring would have to become more equivalent. The former could occur if H-bonding or ion-pairing to the phenolic radical were increased, while the latter could occur upon rotation of the ring.

Photochemistry above 250 K. In contrast to the low-temperature photochemistry, the magnetic susceptibility (χ) given in Figure 5 indicates that light excitation at 273 K produces a normal $S_1 \rightarrow S_2$ state advancement on the first flash, characterized by essentially the same increase in χ compared to a Cl^--reconstituted sample. The data also show that no further changes in χ occur on subsequent flashes, in contrast to Cl^--sufficient samples, indicating that turnover is blocked or that oxidation involves no detectable magnetic change. On the other hand, the EPR experiment given in Figure 2 shows that on the second turnover ($S_2' \rightarrow S_3'$) the 16-mT broad EPR signal forms. This same state can also be reached by continuous illumination at 273 K and trapped by freezing (Figure 1C+E), indicating that further (stable) turnover is blocked at the S_3' oxidation level. This apparent contradiction is addressed in a later section on magnetic susceptibility.

It is possible to determine more quantitatively which S state the 16-mT broad EPR signal is most closely associated with by fitting the flash EPR data to a Kok model. Curve A in Figure 2 is calculated assuming that the signal originates from the S_3 state, the sample is unable to advance past S_3, there is no decay between flashes (5-s interval), there are no other competitive electron donors in their reduced state ($Tyr\text{-}D^+$ is assumed to be fully oxidized), the initial population in the dark is the usual 75% S_1 and 25% S_0 (characteristic of the populations achieved after dark-adapting an illuminated, Cl^--sufficient sample having equal populations of S_0 through S_3), and all centers have the usual 5% double turnovers and 10% misses commonly observed in both PSII particles and core complexes (Sivaraja & Dismukes, 1988). This model is in qualitative agreement with the flash pattern showing a threshold on the second flash, as expected for an S_3 origin. Better agreement with the third flash data is observed if a slightly larger miss parameter is used, or if we include a fraction of centers with a reduced competitive donor. The second trace (curve B) in Figure 2 includes an additional 25% of the centers in the S_0 state to account for $Tyr\text{-}D^+$ decay in Cl^--depleted samples. Competition between photooxidation of the Mn cluster and Tyr-D is normally found in dark-adapted samples (Velthuys & Visser, 1975; Damoder et al., 1986; Styring & Rutherford, 1988). The improved agreement with the data further establishes an S_3 origin for the 16-mT broad signal. Higher S-state assignments are clearly excluded. An S_2-state model with increased miss parameter shows a gradual increase on each flash, which fails to predict the sharp threshold seen between the first and second flashes (not shown).

Our conclusion that F^--substituted samples are blocked from further oxidation beyond the abnormal S_3' state is consistent with earlier work showing that Cl^--depleted samples are also blocked at this state (Ono et al., 1986). The structural change in the Mn cluster which leads to loss of the multiline EPR signal (Casey & Sauer, 1984) does not prohibit Mn oxidation up to the S_2 state. Moreover, this change must be subtle since it leaves unaffected the large increase in the room temperature magnetic susceptibility seen for the $S_1 > S_2$ reaction (Figure 5). Concurrent with these changes we see that electron turnover is blocked beyond the S_3 state at room temperature. This enables us to distinguish between the two proposals cited in the introduction to account for the occurrence of two apparent dissocation constants for Cl^- in the S_1 and S_3 states, seen during Cl^- reactivation of F^--inhibited samples (Damoder et al., 1986). The "tight" and "intermediate" binding constants for Cl^- which we previously reported to be coupled to formation of the S_2 multiline signal and the O_2 evolution step, respectively, most simply reflect a single site which has an 11-fold greater binding affinity in the S_1 state vs the S_3 state. Although the S-state transitions are affected differentially by F^- substitution, there is no need to invoke spatially distinct sites to account for the different binding affinities.

Comparison with Ca^{2+}-Depleted PSII. An EPR signal with essentially identical magnetic properties was recently reported by Boussac et al. (1989) and by us (Sivaraja et al., 1989b) in calcium-depleted PSII membranes which were not depleted of chloride. The isotropic g value, the broad unresolved line width, the saturation, and temperature dependencies for both signals are essentially the same. We thus ask the question whether photooxidation of the species responsible for this signal can be induced by either treatment, or whether the procedure for Cl^- depletion and F^- substitution leads to calcium depletion as a side effect possibly due to the limited solubility of CaF_2.

Besides the spectral similarity of these two signals, there are also essential differences between them: (1) Calcium-depleted membranes form a structurally and kinetically different S_2' state. This possesses a modified multiline EPR signal with narrower ^{55}Mn hyperfine splittings which decays only slowly in the dark over hours. This contrasts with F^--substituted samples for which no multiline signal forms in the S_2' state, and this decays back to S_1' completely within several minutes. (2) The magnetic susceptibility data show a strong increase after one flash in F^--substituted samples (reversible after dark adaptation), while no change is observed in calcium-depleted samples owing to the stability of the modified S_2' state (Tso et al., 1990b). (3) The 16-mT broad signal is also observed, although to a lesser extent (20–60%), in Cl^--depleted membranes which have 15 mM $CaSO_4$ in the medium but no F^-. The lower yield is presumably because of less rigorous Cl^- depletion achieved by mild alkaline washing without a suitable anion available, as previously noted in connection with the O_2 evolution rate and the EPR multiline signal yield (Theg & Homann, 1982; Izawa et al., 1983; Damoder et al., 1986). (4) In F^--substituted samples, the flash study agrees with an S_3'-state model for the new EPR signal, while in Ca^{2+}-depleted membranes two different assignments have been given. Boussac et al. (1989) find that in samples depleted of Ca^{2+} by washing in NaCl/EDTA and rebinding the 18- and 23-kDa proteins, the EPR signal forms in the S_3 state (no flash study was reported). Sivaraja et al. (1989b), using samples depleted of Ca^{2+} by the citric acid/pH 3 procedure, found that a higher state might also be responsible. Recent flash studies from our group have indicated that one or, in some cases, two additional

flashes are required above the S_3 state for formation of the broad $g = 2.00$ signal in Ca^{2+}-depleted samples prepared by citrate extraction (Tso et al., 1990b). The difference in the two types of Ca^{2+}-depleted samples appears to arise from an additional 1 or 2 reducing equiv stored either in the Mn cluster or on Tyr-D^+ in these samples (Sivaraja et al., 1989b; Lockett et al., 1989, 1990). Another striking difference we have noted is the stable photoaccumulation of the 16-mT signal under continuous illumination in F^--substituted PSII membranes vs the photoinhibition which occurs for Ca^{2+}-depleted PSII (Tso et al., 1990c). We thus conclude that the F^- substitution procedure is not a consequence of inadvertent Ca^{2+} depletion. These comparisons lead to several conclusions regarding the mode of inhibition seen with Cl^- depletion vs Ca^{2+} depletion. The structure and kinetic stability of the Mn cluster which is oxidized on the $S_1' > S_2'$ transition differ in F^--substituted and Ca^2-depleted PSII complexes, the latter treatment leading to a greatly stabilized S_2' state and to greater sensitivity to photoinhibition. The number of turnovers that are possible prior to formation of the 16-mT broad signal and hence the number of available electron donors also differ. This signal characterizes the highest oxidation state which has so far been detected under illumination in both samples. Apparently, the Ca^{2+} and Cl^- functions are interdependent.

It was previously proposed that the symmetric 16.3-mT wide signal observed in Ca^{2+}-depleted samples might be due to a radical species in magnetic contact with a transition-metal ion or cluster (Boussac et al., 1989; Sivaraja et al., 1989b). In addition to its spectral similarity with the signal observed in F^--substituted samples, the microwave power dependences are virtually the same. The saturation thresholds are $P_0 = 2.4$ mW (F^-, at 7.9 K), 1.9 mW ($-Ca^{2+}$, citrate/pH 3 treated; at 7.9 K), and 0.8 mW ($-Ca^{2+}$, NaCl/EGTA treated; at 5.9 K; Boussac et al., 1989). The comparison with other types of paramagnetic systems given under Results clearly favors a transition metal or a radical which is spin-coupled to a transition metal or cluster. The latter interpretation is consistent with all the data, while the former view does not fit well with the observed isotropic g value and lack of significant susceptibility change.

Magnetic Susceptibility. The assignment of this species to a radical formed on the $S_2 \rightarrow S_3$ transition leads to the expectation that the magnetic susceptibility should increase by 3 μ_B^2/PSII, while in fact no change is seen in Figure 5. However, this is the maximum increase that could occur and ignores the existence of dead centers which are counted in the total Chl determination of PSII. This is reflected in the spin quantitation which 0.6 spin/Tyr-D^+. Thus, the maximum signal from a free radical to expect would be 1.8 μ_B^2. Figure 2 shows that formation of this radical would also be distributed over four flashes. We believe such a small change could go undetected in our experiment. Moreover, it could be reduced further through coupling to the Mn cluster. Evidence for magnetic contact with the radical is seen in the EPR data. However, it is difficult to predict quantitatively how this will contribute. Even though the Mn cluster appears not to get oxidized on this S-state transition, it is close enough that it could in principle experience a structural change which has magnetic consequences. There are many low-lying magnetic states which contribute to the room temperature magnetic susceptibility of Mn clusters like the one involved in PSII (Dismukes et al., 1982).

Dipole Model. If this proposal is correct, we should be able to predict the distance between the radical and the Mn cluster needed to account for the observed line broadening of the 16-mT broad signal. We shall assume that the observed line width is due to the sum of intrinsic unresolved splittings from the radical (ΔH_r) and the static dipolar splitting it experiences with the Mn cluster (ΔH_d). Since these independent sources must add in quadrature, we have, $\Delta H_{pp}^2 = \Delta H_r^2 + \Delta H_d^2$. Taking 3 mT as a maximum intrinsic line width arising from unresolved hyperfine structure in the radical and $\Delta H_{pp} = 16$ mT, this gives $\Delta H_d = 15.7$ mT. Using the point dipole relationship to relate the maximum dipolar splitting to the distance of separation between the radical and the Mn cluster (Carrington & McLachlan, 1979), and taking isotropic paramagnetic spins states with $S_1 = S_2 = {}^1/_2$, we obtain an approximate distance of 7 Å. The choice of spin $S = {}^1/_2$ for the Mn cluster in the S_2' oxidation is in good agreement with the spin state assignment of the S_2 state in Cl^--sufficient samples, and the magnetic susceptibility results in Figure 5 confirm the same average magnetic moment for F^--substituted samples. The distance of closest approach for this Mn-proximal radical is also greatly restricted by the line-width analysis.

Origin of the Interconversion of the S_2-State EPR Signals. The magnetic data help us to choose between two explanations which have been put forward to account for how the S_2 state may give rise to a normal multiline EPR signal in the native O_2-evolving conformation, while this signal disappears and an unstructured $g = 4.1$ signal replaces it to varying degrees in samples inhibited in O_2 evolution by various treatments (Cl^- depletion, F^- substitution, sucrose versus glycerol solvent, or illumination at 130 K vs >150 K). Aasa et al. (1987) have suggested that the $g = 4.1$ signal may rise from oxidation of a Mn(III) ion to form an isolated Mn(IV) ion which does not interact magnetically with the remaining Mn ions comprising the water-oxidizing complex. Such a model should result in a decrease of the susceptibility by 9 μ_B^2/PSII for high-spin ions, in contrast to observed increase by 6–9 μ_B^2/PSII after one flash (Figure 5). This increase is indistinguishable from that observed upon reconstitution of Cl^-, even though the EPR signals of these two states are very different. We see complete loss of the multiline signal in F^--treated samples, although the formation of the $g = 4.1$ signal is only partial (Figure 1A,D). The lack of formation of the majority of centers in the state producing the $g = 4.1$ signal limits the quantitative conclusions we can draw from this experiment. The qualitative result is clear, however. F^- substitution cannot involve large changes in the bridging ligand structure of the Mn cluster in the S_2 state nor a change in the site of oxidation to an isolated Mn ion. The magnetic susceptibility of polynuclear manganese clusters is determined by the inter-ion exchange interaction and so is a sensitive function of the bridging ligand geometry (Wieghardt, 1989; Christou, 1989). These data support our earlier model in which the large increase in magnetic susceptibility observed on the $S_1 \rightarrow S_2$ transition can be attributed to the simultaneous one-electron oxidation and magnetic uncoupling of a tri- or tetranuclear Mn cluster (Sivaraja et al., 1989a), in agreement with earlier EPR studies (Dismukes & Siderer, 198a; Dismukes et al., 1982). This also fits in nicely with the alternative proposal given to account for the EPR results in which both the multiline and $g = 4.1$ signals arise from a single tetramanganese cluster which, through a conformational change, can have as its ground state either an $S = {}^1/_2$ or an $S = {}^3/_2$ spin state, respectively (dePaula et al., 1986; Zimmermann & Rutherford, 1986).

A Proposal for the Molecular Mechanism of Inhibition of O_2 Evolution by Cl^- Depletion and by Ca^{2+} Depletion. It is reasonable to conclude that both Cl^- and Ca^{2+} interact with a common site within the water-oxidizing complex such that

depletion of either induces the same response to photooxidation. This site would appear to be either the Mn cluster or Tyr-Z^+. The observation that the structure of the Mn cluster is visibly perturbed in the S_2 state and becomes thermodynamically stabilized against recombination with Q_A^- in the S_2 state by both treatments (Ono & Inoue, 1990; Ono et al., 1986a, 1987; Homann et al., 1986; Vass et al., 1987; Rosza & Demeter, 1987) is evidence which favors the Mn cluster as being the common locus of inhibition. We presume that stabilization of the S_2 oxidation state relative to the S_3 state is responsible for the abortive photooxidation of an endogenous cofactor, most likely an amino acid residue in magnetic contact with the Mn cluster. We consider next how this could occur.

The chemical origin of the common changes observed upon Cl^- depletion, F^- substitution, and Ca^{2+} depletion can be understood in terms of the known chemistry of synthetic manganese clusters. Both redox stabilization of the Mn(IV) oxidation state and valence trapping of mixed-valence oxidation states of the Mn cluster in PSII would have profound consequences on the ability of Tyr-Z^+ to oxidize the cluster and hence on O_2 evolution. How the first of these could occur is obvious. If the reduction potential of the cluster decreases, it can still be oxidized by Tyr-Z^+, but water oxidation will be suppressed if the potential drops below 0.82 V. On the other hand, if valence trapping occurs so that the special "gateway" Mn ion becomes stabilized in the Mn(IV) oxidation state upon photooxidation of $S_1' \rightarrow S_2'$, then Tyr-Z^+ may be unable to further oxidize the cluster since this would require oxidation to Mn(V) at this site. The "gateway" Mn ion should be the one which mediates intramolecular electron transfer between Tyr-Z^+ and the other species involved in charge accumulation, normally presumed to be the three other Mn ions, but see below. Strong valence trapping of the manganese ions is responsible for the characteristic multiline EPR signal seen in PSII (Dismukes & Siderer, 1981) as well as the EPR signals for all of the reported dimanganese(III,IV) complexes (Dismukes et al., 1987) and II,III complexes (Chang et al., 1987) reported to date. Nonexchanging, deeply trapped oxidation states of Mn(III) and Mn(IV) have been observed in all of the X-ray structures reported to date for mixed-valence Mn clusters, of which there are many (Wieghardt, 1989; Cristou, 1989; Pecoraro, 1988; Chang et al., 1987). We therefore suggest that valence trapping of the special gateway Mn ion in the Mn(IV) oxidation state on the $S_1' \rightarrow S_2'$ transition offers a plausible explanation for how the effects of both Cl^- depletion and Ca^{2+} depletion could express a common outcome in terms of abortive oxidation of a Mn-proximal radical.

Relationship to Normal Water Oxidation Chemistry. What does this study say about the mechanism of water oxidation in PSII? Formation of the Mn-proximal radical in the S_3' state establishes that the site of charge accumulation need not be localized exclusively to Mn ions on each of the S-state transitions in inhibited samples. What about the normal S_3 state? Most mechanisms have presumed that the four Mn ions found in PSII are needed for accumulating the 4 oxidizing equiv essential for water oxidation. While this may yet be proven true, evidence from Mn near-infrared electronic absorption (Dismukes & Mathis, 1984), EPR (Styring & Rutherford, 1988), and Mn X-ray absorption (Guiles et al., 1990) suggests that something other than Mn seems to be oxidized by one electron on the $S_2 \rightarrow S_3$ transition in native O_2-evolving centers. This species would have to be strongly coupling and hence directly coordinated to the Mn cluster to account for the loss of the S_2 multiline EPR signal in the S_3 state (Dismukes & Siderer, 1981). Dissociation of this species from the Mn cluster upon Cl^- depletion or Ca^{2+} depletion might then account for the appearance of the Mn-proximal radical EPR signal which we report here.

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Registry No. O_2, 7782-44-7; Cl^-, 16887-00-6; F^-, 16984-48-8; Mn, 7439-96-5.

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