Ca^{2+} Depletion from the Photosynthetic Water-Oxidizing Complex Reveals Photooxidation of a Protein Residue†

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ABSTRACT: A new intermediate in the water-oxidizing reaction has been observed in spinach photosystem II (PSII) membranes that are depleted of Ca^{2+} from the site which is conformationally coupled to the manganese cluster comprising the water-oxidizing complex (WOC). It gives rise to a recently identified EPR signal (symmetric line shape with width 163 ± 5 G, $g = 2.004 \pm 0.005$), which forms in samples inhibited either by depletion of Ca^{2+} [Boussac, A., Zimmerman, J.-L., & Rutherford, A. W. (1989) *Biochemistry 28*, 8984–8989; Sivaraja, M., Tso, J., & Dismukes, G. C. (1989) *Biochemistry 28*, 9459–9464] or by substitution of Cl^- by F^- (Baumgarten, Philo, and Dismukes, submitted for publication). Further characterization of this EPR signal has revealed the following: (1) it forms independently of the local structure of the PSII acceptors; (2) it arises from photooxidation of a PSII species that donates an electron to Tyr-Z^+ or to the Mn cluster in competition with an exogenous donor (DPC); (3) the Curie temperature dependence of the intensity suggests an isolated doublet ground state, attributable to a spin $S = {}^1/_2$ radical; (4) the electron spin orientation relaxes 1000-fold more rapidly than typical for a free radical, exhibiting a strong temperature dependence of $P_{1/2}$ (half-saturation power $\sim T^{3.4}$) and a broad inhomogeneous line width; (5) it yields an undetectable change in the magnetic susceptibility upon formation by a laser flash; (6) it disappears in parallel with release of Mn during reduction with NH_2OH, indicating that it forms only in the presence of the modified Mn cluster. One possible interpretation attributes this EPR signal to an unusual state of the Mn cluster not previously observed in synthetic Mn_2, Mn_3, or Mn_4 clusters. However, the most likely possibility is a radical which is in dipolar contact with a rapidly relaxing spin center such as a transition ion. We attribute this to photooxidation of an amino acid residue located within 9 Å of the Mn cluster. Starting in dark-adapted samples exhibiting the modified multiline EPR signal (apparent S_2' oxidation state), the 160-G EPR signal forms with variable yield (24–48%) on the first flash and in >80% centers after two saturating flashes. It thus appears associated with both the S_3' and S_4' states. This signal decays in the dark to the S_2' state. The radical signal slowly disappears upon multiple turnovers during continuous illumination and may be correlated with the onset of photoinhibition—the irreversible loss of O_2 evolution. Photoinhibition occurs at a 17-fold faster rate than in normal PSII centers. We consider evidence suggesting that photooxidation of this species may occur during the normal mechanism of water oxidation.

Photosynthetic organisms are inactivated in strong light by a process termed photoinhibition (Powles, 1984). It is an important source of crop damage, especially under conditions of environmental stress. It is triggered by pigment photochemistry occurring in photosystem II (PSII)[1] which initiates damage of the D_1 subunit of the reaction center (Q_B binding protein; Kyle et al., 1984) and may also involve the D_2 subunit (Callahan et al., 1986). Repair of this damage requires protein synthesis and reassembly of the PSII complex. Several of the reactions in PSII have been proposed as the locus for this inhibition, including abnormal oxidation reactions of electron donors, primary photochemistry, Q_B reduction, and double reduction of Q_A (Kyle et al., 1984; Allakhverdiev et al., 1987; Vass et al., 1988; Demeter et al., 1987; Callahan et al., 1986; Styring et al., 1990). Under conditions of stress, including Cl^- depletion or complete removal of the WOC, photoinhibition occurs at lower light intensities and may proceed via a different mechanism. Under these conditions, the site of inhibition is on the donor side principally between Tyr-Z Tyr-Z and the Mn cluster (Callahan et al., 1986; Jegerschold & Styring, 1989), or between P680 and Tyr-Z (Theg et al., 1986). Photoinhibition of PSII centers stripped of the water-oxidizing complex changes the environment and suppresses photooxidation of the normally functional Tyr-Z^+ and Tyr-D^+ radicals, while inducing formation of another free radical (Blubaugh & Cheniae, 1989). The latter has been attributed to formation of a Chl^+ radical close to the reaction center (Thompson & Brudvig, 1988).

Here we show that the increased rate of photoinhibition in Ca-depleted PSII is associated with a structural modification of the Mn cluster and that formation of a radical is precursor to inhibition. This radical has been characterized by EPR in recent work (Sivaraja et al., 1989b; Boussac et al., 1989). One of the unresolved issues of this earlier work is the oxidation state in which the signal forms. Boussac et al. (1989) assigned this to a modified S_3' state in samples depleted of calcium by NaCl/EDTA washing. We reported (Sivaraja et al., 1989b) the signal to originate from an oxidation state at or above the S_3' level in samples depleted of calcium using the low-pH/

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[1] Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; DPC, diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; WOC, water-oxidizing complex.

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citrate method developed by Ono and Inoue (1988).

Earlier work has also left unresolved the more general question of the number of light-induced turnovers which can occur in calcium-depleted membranes. From EPR studies of the S_2-state multiline signal in salt-washed membranes, Blough and Sauer (1984), dePaula et al. (1986), and Kalosaka (1990) deduced that the first turnover corresponding to the $S_1 \rightarrow S_2$ transition is blocked. In contrast, delayed fluorescence (Boussac et al., 1985), thermoluminescence (Ono & Inoue, 1986), and EPR studies (Boussac & Rutherford, 1988) consistently indicated that the S_3 to S_0 transition is inhibited. From thermoluminescence studies, Ono and Inoue (1988, 1989a) concluded that low-pH citrate-treated PSII membranes are unable to advance beyond the S_2 state. We suggest that a possible source of these discrepancies is the presence of an additional reductant on the donor side in calcium-depleted membranes prepared by this method (Sivaraja et al., 1989b) and also in salt-washed membranes (Lockett et al., 1990).

Materials and Methods

Sample preparation, biochemical characterization, and EPR measurements were performed as described previously (Sivaraja et al., 1989b; Tso et al., 1991). Formate treatment was done according to Vermaas and Rutherford (1984).

The 160-G-wide signal was generated by illumination for 20 s at 273 K of calcium-depleted samples in 4-mm quartz tubes at a chlorophyll concentration of 5 mg/mL and light intensity 2 W/cm^2 from a quartz–halogen source, except where otherwise specified.

For magnetic susceptibility measurements, samples were given a single preflash and dark-adapted for 15 min prior to giving the sequential train of flashes. Magnetic susceptibility was measured at room temperature according to the procedure outlined by Sivaraja et al., (1989a). Prior to susceptibility measurements, the samples were flushed with N_2 gas for 10 min to remove dissolved O_2. Typically, a train of six laser flashes of 20–50 mJ/pulse from a Candella SLL 625 dye laser (570 nm, 500-ns pulse width) was delivered to the sample through a 2-mm core diameter fiber optic capable every 2.0 s. This is sufficient intensity to ensure light saturation at the concentration used. The sample volume was approximately 225 μL. Each data set is an average of two experiments. The susceptibility data have been corrected for the unavoidable increase in the diamagnetism of the sample during each laser pulse, proportional to the laser energy, due to heating (<0.03 °C) and the temperature dependence of the diamagnetism of water. These heating artifacts are less than 10% of the peak photochemical signals. Data were recorded with a 300-ms time constant filter to improve the signal/noise ratio. The magnetometer probe has an intrinsic mechanical resonance at 1.3 Hz, and vibration-induced noise at this frequency appeared in some of the data. This noise was removed during post-processing by digital filtering techniques which have a negligible effect on the tube photochemical signals.

Results

Yield of the 160-G-Wide EPR Signal as a Function of Illumination Time. Figure 1 shows that there is an optimum illumination time that exists for formation of the 160-G-wide EPR signal in calcium-depleted PSII. A maximum is reached after about 20-s illumination time for this light intensity, and the signal decays with further illumination. The decay could be due to further photooxidation beyond the state giving rise to the 160-G-wide signal or due to photodamage. The loss of the EPR signal under continuous illumination is not accompanied by recovery of O_2 activity. The yield of the signal

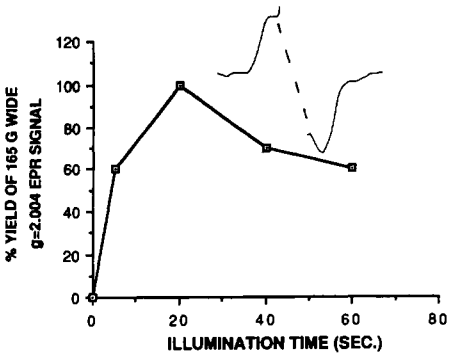


FIGURE 1: Yield of the 160-G-wide EPR signal in calcium-depleted PSII membranes as a function of illumination time at 273 K. The samples were illuminated and immediately frozen to 77 K. One hundred percent yield equals 1–2 spins/PSII. Sample buffer: 400 mM sucrose, 10 mM NaCl, and 10 mM MES (pH 6.5). The electron acceptor DCBQ (0.5 mM) was used. Chl concentration = 5 mg/mL. EPR conditions: sample temperature = 8 K; microwave power 32 mW; modulation amplitude = 20 G.

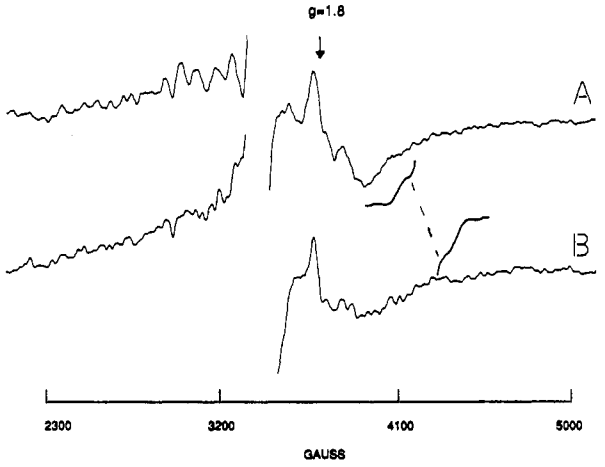


FIGURE 2: EPR spectra of control (A) and calcium-depleted (B) PSII membranes treated with 100 mM formate (as described under Materials and Methods) and illuminated at 273 K for 20 s and frozen immediately to 77 K. Conditions same as for Figure 1, except T = 4.2 K. Inset, T = 8 K.

at its maximum corresponds to 1–2 spins/PSII (Sivaraja et al., 1989b).

160-G-Wide EPR Signal Arises from a PSII Donor. We consider three possibilities for the origin of the 160-G-wide signal: (1) a donor species that is created by photooxidation beyond the S_2' state; (2) an altered FeQ_A^- species appearing at g = 2.0 instead of the normal g = 1.9 or g = 1.82 signals; (3) a semiquinone arising from either bound $DCBQ^-$ or an altered FeQ_B^-. We first consider the third possibility. Because the signal forms in 10% yield in the absence of an exogenous quinone, we conclude that it cannot be due solely to the semiquinone of DCBQ [Figure 2B of Tso et al. (1991)]. It forms maximally in samples containing an exogenous quinone needed to replace plastoquinone lost during isolation of PSII membranes (this displaces endogenous Q_B). Thus, efficient charge separation is a requirement.

To check the second possibility, we looked in Ca-depleted samples for formation of the normal FeQ_A^- EPR signals appearing at g = 1.9. Unfortunately, we are unable to directly look for formation of this signal due to masking of this region by the large 160-G-wide signal. In order to overcome this problem, the g = 1.9 signal can be converted to the g = 1.82 form by pretreatment with 100 mM formate (Vermaas & Rutherford, 1984). In Figure 2, we compare Ca-sufficient (A) to Ca-depleted (B) PSII membranes containing 100 mM

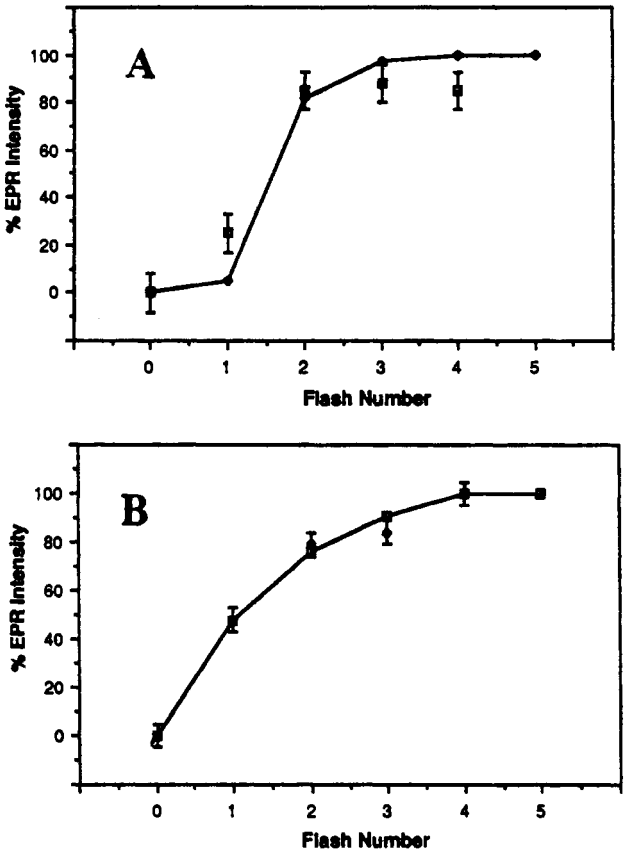


FIGURE 3: Yield of the 160-G-wide EPR signal upon stepwise laser flash turnover of calcium-depleted PSII membranes. Two data sets are given. The samples were poised in the S_2' state (as described in the text) prior to flash turnover. The solid lines give fits to Kok models described in the text. Chl concentration = 0.9 mg/mL. Other conditions as in Figure 1.

formate, which were illuminated at 273 K and frozen to 77 K. The majority of the FeQ_A^- centers in both samples now exhibit the $g = 1.82$ form. The intensity of the 160-G-wide signal is nearly the same as that observed in the control Ca-depleted samples (see insert to Figure 2B). This indicates that the 160-G-wide signal is not another form of the FeQ_A^- EPR signals.

This suggests that the 160-G-wide signal may arise from photooxidation of a reaction-center donor species. If true, then addition of an exogenous donor such as diphenylcarbazide (DPC) might compete for oxidation by the reaction-center tyrosine-Y_Z^+. We found that addition of 2 mM DPC before illumination reduces the intensity of the 160-G-wide signal to 70%, while 4 mM DPC reduces the signal to about 60% of the maximum (not shown). These data indicate that photooxidation of the 160-G-wide signal occurs in competition with electron donation from DPC to Y_Z^+. Because dislocation of Mn from its native site is a prerequisite for electron donation from DPC to Tyr-Z^+ to be observed, this result suggests that a structural change occurs upon Ca^{2+} depletion. Addition of NH_2OH also reduces the yield of the 160-G-wide $g = 2.0$ signal, in parallel with release of manganese (not shown). This indicates that this signal forms upon photooxidation of a center only in the presence of the modified Mn cluster.

All of these data point to an origin for the 160-G-wide signal as a reaction-center electron donor.

Single Turnovers of Ca-Depleted PSII Membranes. Dilute Ca-depleted PSII samples were illuminated at 273 K with a train of laser flashes of saturating intensity from a Nd-YAG laser (532 nm, 10-ns pulse width, 1-Hz repetition rate), and the yield of the 160-G-wide EPR signal was monitored after

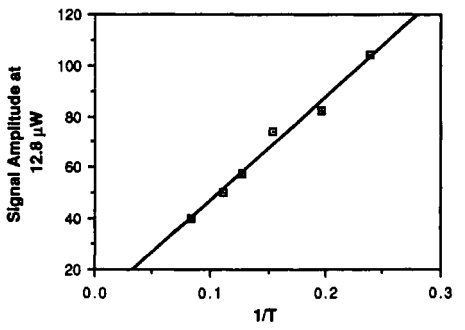


FIGURE 4: Temperature dependence of the 160-G-wide $g = 2.0$ EPR signal intensity. The amplitude of the signal measured at 12.8 μW is plotted against $1/T$ (K^{-1}). The temperature ranges from 4.2 to 9 K. Conditions same as in Figure 1.

quench-cooling to 77 K. Prior to flash illumination, the Ca-depleted samples were poised in the dark-stable S_2' state reached by preillumination at 273 K and dark-adaptation for 15 min, as previously shown (Sivaraja et al., 1989b; Boussac et al., 1989). Figure 3 shows the results from two of five experiments done with different batches of calcium-depleted PSII membranes. One hundred percent yield is defined to be the yield produced by CW illumination (as in Figure 1).

There is a qualitative difference in the flash pattern obtained for the two data sets. The largest increase in signal intensity is induced on the second flash (from 24% to 82%) in the first data set in panel A, while for the sample in panel B a comparable increase is distributed over the first and second flashes (from 0% to 48% and from 48% to 83%, respectively). This variability is not due to experimental error but rather due to an inherent variability in the samples. The average of four data sets gave increases of 38% on the first flash and 79% on the second flash. The first data set was found to fit a Kok model for the S_4' population (Figure 3A, solid line), using as parameters α(misses) and = 10% and β(double hits) = 5% and assuming a block in turnover at S_4'. These are the usual miss and double-hit parameters found in the control PSII membranes. The second data set was found to fit a Kok model for the S_3' population (Figure 3B, solid line), using as parameters α = 43% and β = 5% and assuming a block in turnover at S_3'.

This sample variability appears to be due to formation of a reduced center on the donor side by the Ca-depletion procedure. Independent evidence for reduction of the water-oxidizing complex by Ca depletion has been seen by the transfer of this reducing equivalent to Tyr-Y_D^+ upon calcium reconstitution to material depleted of calcium either by the citrate extraction method (Sivaraja et al., 1989b) or by salt washing (Lockett et al., 1989). In the calcium-depleted state, the site of the reduction has not been clearly established.

Temperature Dependence of the 160-G-Wide EPR Signal. A plot of the amplitude of the 160-G-wide signal as a function of $1/T$ at a microwave power well below saturation exhibits a linear dependence indicative of Curie behavior (Figure 4). This implies that the paramagnet involves only a two level system without population of excited spin states in the temperature region studied (4.2–12 K). Such behavior is typical of a simple $S = {}^1/_2$ spin system. A strong candidate for such behavior is a free radical species. However, the large peak-to-peak line width is much larger than any simple free radical, which typically exhibit spectral widths less than 30 G, due primarily to unresoled hyperfine couplings to protons and nitrogen nuclei.

Figure 5 shows the temperature dependence of $P_{1/2}$, the microwave power required for half-saturation, plotted as ln

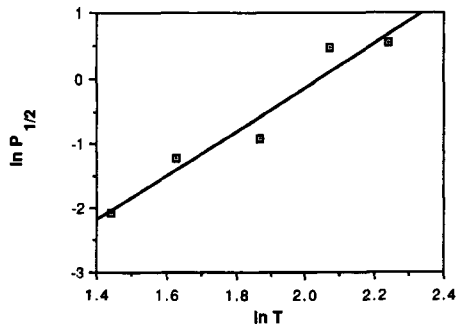


FIGURE 5: Plot of ln $P_{1/2}$ versus ln T for the 160-G-wide $g = 2.0$ EPR signal. Conditions as in Figure 1. $P_{1/2}$ = half-saturation power.

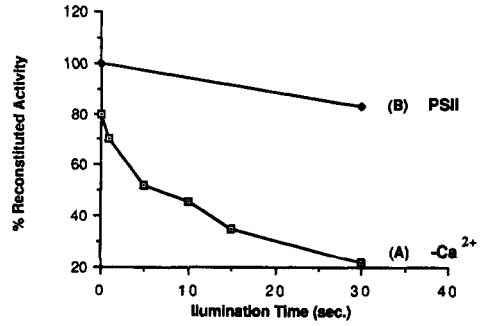


FIGURE 6: Photoactivation of calcium-depleted PSII membranes. O_2 evolution rate versus preillumination time. Calcium-depleted samples were preilluminated for different lengths of time followed by dark readaption for 15 min, and treated with 50 mM $CaCl_2$ prior to an additional dark incubation for 15 min. Chlorophyll concentration = 40 μg/mL. Sample buffer as in Figure 1.

$P_{1/2}$ against ln T. $P_{1/2}$ at each temperature was calculated graphically from a plot of log (intensity/$\sqrt{P}$) versus log P (Yim et al., 1982). The data can be fitted to the expression ln $P_{1/2} = -6 + 3.4$ ln T, implying that $P_{1/2}$ varies strongly with temperature. At and above 12 K, we are unable to saturate the signal with 200 mW of power. This indicates that although the spin population follows the simple Curie temperature dependence typical of a two-level Kramers system (e.g., a $S = {}^1/_2$ particle), it also exhibits strong relaxation, which is not expected for a simple (isolated) spin $S = {}^1/_2$ system and more typical of transition-metal ions or $S > {}^1/_2$ systems.

Photoinhibition of Ca-Depleted PSII Membranes. Susceptibility to irreversible photodamage has been shown to be greatly enhanced in chloroplasts and PSII membranes inhibited on the donor side either by Cl^- depeletion or by Tris tratment to release the extrinsinc proteins and manganese (Critchley et al., 1984; Theg et al., 1986). A similar photodegradation phenomenon can be seen in Ca-depleted samples. Ca-depleted samples at a low chlorophyll concentration of 40 μg/mL were preilluminated for various lengths of time in the presence of DCBQ, dark-adapted for 15 min, then incubated with 50 mM $CaSO_4$ for 10 min, and assayed for O_2 evolution activity. Figure 6 is a plot of the percent O_2 evolution activity reconstituted as a function of the preillumination time prior to Ca reconstitution. With no preillumination, about 70% O_2 evolution activity is recovered. After 30-s illumination, only 20% of activity is recovered, whereas illuination of an untrated sample for the same length of time results in less than 15% loss in O_2 evolution activity. There is a 17-fold increase in the initial rate of photoinhibition. If an electron acceptor is not present during the illumination, there is almost no photodegradation of the Ca-depleted samples during the 30-s illumination (not shown). This result implies that multiple turnovers are needed for the damage to occur. Because the 160-G-wide signal photoaccumulates during the preillumination (Figure 3) and decays in the dark to the stable S_2', we conclude that the S state giving rise to this signal is a precursor to photoinhibition.

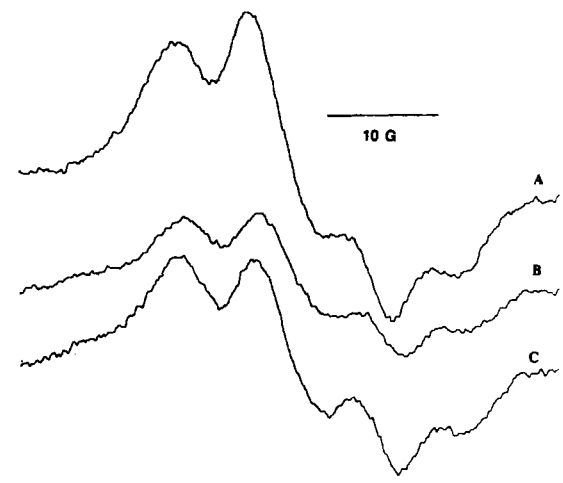


FIGURE 7: Effect on signal II_S of calcium reconstitution to Ca^{2+}-depleted PSII membranes: (A) dark-adapted untreated PSII membranes (control). Ca^{2+} reconstitution to (B) the S_2' state and (C) the state giving rise to the 160-G-wide signal. Both samples were dark-adaptated for 30 min after calcium reconstitution. EPR conditions: temperature = 8 K; microwave power = 0.2 mW; modulation amplitude = 0.5 G. Other conditions as in Figure 1.

Ca^{2+} Reconstitution. We (Sivaraja et al., 1989b) and Lockett et al. (1989, 1990) have shown that calcium reconstitution to samples poised in the S_1' or S_2' states leads to the reversible reduction of EPR signal II_S (reduction of the stable tyrosine donor Y_D^+) over a period of several minutes. This indicated the presence of a hidden reductant which reacted with Y_D^+ upon Ca reconstitution.

Figure 7C shows the effect on signal IIs of calcium readdition to samples that were preilluminated at 273 K in order to generate the higher S state responsible for the 160-G-wide EPR signal. Calcium was added and mixed within 10 s of the end of illumination, which is much less than the decay time of the signal in the absence of calcium. The sample was then incubated in the dark for various times, and the yield of EPR signal II_S was compared with that in control PSII membranes (Figure 7a). A second comparison is made to a sample in which calcium was reconstituted to depleted membranes in the S_2' state and dark-adapted for 30 min (Figure 7B). In trace B, there is about a 50% loss in signal II_S relative to the control (trace A), which is consistent with our earlier results (Sivaraja et al., 1989b). In curve C, about 80% of signal II_S is retained after a 30-min dark-adaptation period. This result shows that the state giving rise to the 160-G-wide signal is less capable of reducing Y_D^+ following calcium readdition than is the precursors state S_2'. We interpret this as due to oxidation of a reducing equivalent formed during Ca depletion in the dark. Another possibility, that calcium does not rebind in this state, can be discounted on the basis of the reactivation of O_2 activity and the S_2 multiline signal, as previously reported by Boussac et al. (1989).

Magnetic Susceptibility. Figure 8 shows the magnetic susceptibility changes created by a train of six laser pulses given to (A) control PSII membranes which are O_2-evolving, (B) calcium-depleted PSII, and (C) calcium-reconstituted PSII membranes. The data are given in terms of the change in squared effective magnetic moment ($\Delta\mu_{eff}^2$), in units of squared Bohr magnetons per PSII, based on the measured total chlorophyll concentration and assuming 225 Chl/PSII. All three samples were given a preflash and dark-adapted for at least 15 min prior to a train of six measurement flashes. For

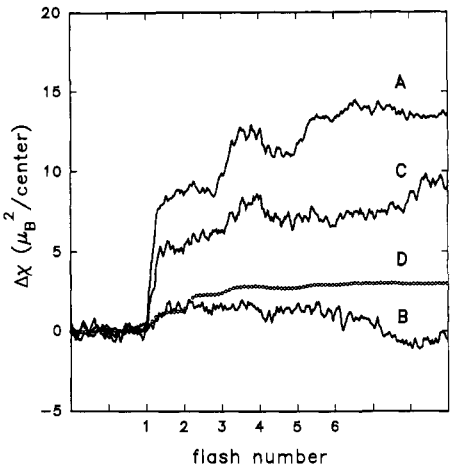


FIGURE 8: Flash-induced magnetic susceptibility changes of (A) untreated, (B) Ca^{2+}-depleted, and (C) Ca^{2+}-reconstituted PSII membranes. The samples were given a preflash and then dark-adapted for at least 20 min prior to the flash measurements. Chl concentration = 1 mg/mL; 1–2 mM DCBQ; sample buffer same as in Figure 1. (D) is a theoretical cureve for the Ca-depleted PSII assuming (1) the 160-G-wide EPR signal is due to one free radical per PSII and has the flash dependence given by Figure 3A, (2) there are no magnetic changes from the Mn cluster, and (3) 10% of the centers have retained Ca^{2+} and 30% are inactive (details in text).

samples A and C, the preflash synchronizes the PSII centers in the S_1 state, while for sample B this places all centers in the modified S_2' state. The latter occurs in Ca-depleted samples owing to the increased thermodynamic and kinetic stability of this state (Ono & Inoue, 1989a,b; Sivaraja et al., 1989b; Boussac et al., 1989).

All of the changes in the magnetic susceptibility seen in the control PSII (curve A) were previously shown to be due to increased paramagnetism arising from the reactions of the water-oxidizing complex (Sivaraja et al., 1989a). By contrast, the calcium-depleted samples in curve B show little change in magnetic susceptibility on any of the flashes. When calcium is reconstituted to the sample (curve C), a pattern similar to that of the undepleted control is obtained, but with about 70% yield on all flashes. This recovery is consistent with the 70% recovery observed in O_2 evolution activity.

For the Ca-depleted sample, the small signal on the first flash can be attributed to about 10% of centers which have not lost calcium, on the basis of residual O_2 evolution activity. Thus, there is no significant change in the magnetic susceptibility on the first flash for the $S_2' \rightarrow S_3'$ transition, even though the elimination of the modified S_2' multiline EPR signal tell us that oxidation of a donor species has occurred. If the 160-G-wide signal is due to formation of a radical (susceptibility 3 μ^2), one would expect to see an increase in paramagnetism on the second and third flashes as this species is formed. Such a change may be difficult for us to observe, however, given that it is spread over several flashes, and because the sample contains ~30% inactive center and ~10% of centers that retain Ca^{2+}. For illustrative purposes, we have calculated curve D to show the total signal expected in this sample from the formation of (1) free radical per center, with the flash dependence we observed in Figure 3A, as well as the residual signal from the calcium-containing centers. That is, curve D is calculated by assuming that there are no susceptibility changes from the Mn cluster in the Ca-depleted material. The lack of changes after the first flash in curve C therefore suggests that there may be small decreases in the susceptibility of the Mn cluster which mask the formation of the radical. However, given our present sensitivity, the difference between curves C and D is of marginal significance.

Discussion

Evidence That the 160-G-Wide EPR Signal Arises from a Donor Species. We attribute the 160-G-wide EPR signal to oxidation of an endogenous protein-bound donor to the PSII reaction center based on the following: (1) the signal decreases upon addition of a competitive electron donor (DPC); (2) it occurs in stoichiometric proportion to the reaction center (1–2 spins/Tyr-D^+) (Sivaraja et al., 1989b); (3) it is abolished by prior release of manganese by treatment with NH_2OH (Tso et al., 1991). An alternative assignment as an electron acceptor within PSII does not fit with the following observations: (1) The yield of the 160-G-wide signal is independent of the spectral form of the EPR signal for FeQ_A^- (both the $g = 1.82$ and $g = 1.9$ forms); (2) the yield of these latter signals is unaffected by Ca depletion.

S-State Origin. The 160-G-wide signal was previously assigned to an S state above S_2' on the basis that it decays to the S_2' state in the dark, yielding the modified multiline EPR signal (Boussac et al., 1989; Sivaraja et al., 1989b). The EPR studies by Boussac et al. (1989) further assigned this signal as originating in the S_3' state owing to its decay upon Ca readdition to form a normal S_2 multiline signal. From this and thermoluminescence studies (Boussac & Rutherford, 1988), they have deduced that the state responsible for the 160-G-wide signal must be a modified S_3' state. Their results have been obtained in Ca-depleted membranes prepared by NaCl/EDTA washing. Our results differ with this conclusion, in that one additional turnover is needed for maximum formation of this signal, but apply to Ca depletion by low-pH/citrate washing. We have consistently observed this, for samples undergoing turnover both by room temperature flashes (Figure 3) and by 195 K illuminations (Figure 4; Tso et al., 1991). This discrepancy with the results of Boussac et al. (1989) may be due to the difference in Ca-depletion methods. However, this and the variability we see in the flash response on different samples may also be due to the presence of an additional reductant in the dark within or accessible to the water-oxidizing complex in the low-pH/citrate-treated samples. If up to 1 full reducing equiv is assumed to be present, then the S-state assignment in our work for the signal producing the 160-G-wide EPR signal would be S_3'.

We (Sivaraja et al., 1989b) and Lockett et al. (1989, 1990) previously showed that a hidden reductant exists within the Ca-depleted PSII complex which can be revealed by its reduction of Tyr-D^+ upon Ca^{2+} reconstitution in the dark. The experiments reported by Lockett et al. used salt-washed PSII membranes which were also depleted of the 17- and 23-kDa extrinsic proteins. In Figure 7, we showed that prior photooxidation to form the 160-G-wide signal suppresses the extent of subsequent Tyr-D^+ reduction which can be observed upon Ca reconstitution. The origin of the changes responsible for this apparent reduction of the water-oxidizing complex remains unresolved. However, we have suggested that it may be due to disproportionation of the Mn cluster induced by hydrolysis, in analogy to the reaction of synthetic Mn clusters in water (Sivaraja et al., 1989b).

Spin Relaxation Mechanism of the 160-G-Wide Signal. The efficient spin relaxation evident by the high values observed for $P_{1/2}$ and its strong temperature dependence (proportional to $T^{3.4}$) (Figure 5) are not expected for a simple doublet spin system, as is predicted from the Curie behavior of the EPR intensity and the isotropic g value. Theory predicts that $P_{1/2}$ should depend on both the longitudinal and transverse spin relaxation times, T_1 and T_2. The dominant spin relaxation

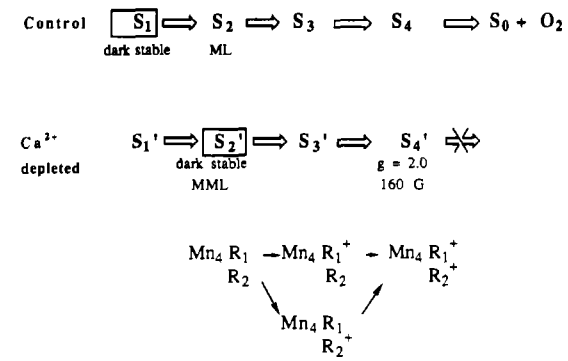
pathway cannot be the dirct one-phonon T_1 process, since this would imply a simple linear dependence of $P_{1/2}$ on temperature. Higher order temperature-dependent processes include Orbach or multiphonon Raman T_1 processes or cross-relaxation processes. The Orbach process can be eliminated because this requires the presence of low-lying excited states, which would produce a non-Curie temperature dependence of the signal amplitude, contrary to our observations (Figure 4). For the multiphonon Raman process, $P_{1/2}$ generally has a T^3 to T^7 dependence, which is consistent with what we observe (Abragam & Bleaney, 1970). The cross-relaxation mechanism dominates whenever a slowly relaxing spin such as a free radical is coupled to a rapidly relaxing spin. This can involve either direct or indirect dipolar interaction (Pake & Estle, 1973). An example of an efficient relaxer would be a nearby transition ion such as the Mn cluster. This mechanism is the most consistent with our results because it enables explanation of both the Curie temperature dependence of the EPR intensity, suggesting a spin $S = {}^1/_2$ ground state, and rapid relaxation as seen via $P_{1/2}$ and its temperature dependence.

Magnetic Susceptibility of Ca-Depleted PSII. The lack of significant light-induced changes in the magnetic susceptibility of Ca-depleted PSII on the first flash indicates that there is no measurable change in the Mn cluster on the $S_2' \rightarrow S_3'$ transition. The susceptibility of the cluster depends on both the spin state of the ions (which *must* decrease upon oxidation for high-spin electron configurations common to Mn^{3+} and Mn^{4+}) and the strength of the intracluster exchange interactions between the ions (which in model compounds also vary strongly with oxidation state). Therefore, one interpretation of this result would be that the Mn cluster is not oxidized on this transition. However, previous results showed that the modified multiline EPR signal disappears on this $S_2' \rightarrow S_3'$ oxidation step (Sivaraja et al., 1989b; Boussac et al., 1989), which most likely indicates oxidation of the Mn cluster or a ligand coordinated to it. If Mn is oxidized, there must be compensating changes in exchange interactions to give no appreciable change in susceptibility. If a coordinating ligand is oxidized to a radical, the lack of susceptibility change requires this radical to be magnetically coupled to the cluster (which is consistent with the EPR silence of the S_3' state).

The absence of significant susceptibility changes ($<1\ \mu^2$) on the second and third flashes in Ca-depleted PSII indicates that there are no substantial changes in the susceptibility of the Mn cluster on the $S_3' \rightarrow S_4'$ transition. The susceptibility data do not clearly show the formation of a new $S = {}^1/_2$ free radical as the 160-G-wide EPR signal forms on the second flash. However, oxidation of a nearby group could easily produce compensating changes in the Mn cluster exchange interactions which could offset this. Therefore, the susceptibility data are not inconsistent with a radical origin for the 160-G-wide EPR signal.

Chemical Origin of the 160-G-Wide EPR Signal. We consider next two possibilities for the identity of the 160-G-wide EPR signal: either the Mn cluster or an amino acid radical in dipolar contact with a transition-metal center. The lack of any hyperfine structure on the signal generally rules out simple odd-spin Mn clusters. For even-spin clusters, the ^{55}Mn hyperfine splitting is reduced compared to the monomer splitting. For homovalent clusters in the highest spin state, this reduces the splitting by a factor of N, where N = the number of Mn ions in the cluster (Dismukes et al., 1982). Thus, for a tetranuclear cluster, the reduction is one-fourth, which could obscure resolution of structure if anisotropies were also present. Wieghardt et al. (1983 have reported a symmetric tetranuclear cluster, $Mn_4O_6L_4$, with L = 1,4,7-triazacyclononane, exhibiting a featureless symmetric 400-G-wide EPR signal centered at $g = 2.0$. The oxo-bridged Mn(IV) ions are weakly coupled ferromagnetically to yield a ground electronic spin of $S = 8$ with many low-lying spin states. Protonation of one oxo gives a weak antiferromagnetically coupled cluster (Hagen et al., 1989). We have examined the temperature dependence of this EPR signal and observed the expected non-Curie dependence (Dismukes, unpublished results). Although the EPR g value is similar to those of the signal we see in PSII, its non-Curie temperature dependence is incompatible with this signal. Moreover, oxidation of Mn to form a ferromagnetic cluster should have created a large increase in the magnetic susceptibility, but no increase was observed, as shown in Figure 8.

Scheme I



These observations favor the second possibility in which the 160-G-wide signal arises from photooxidation of an organic species, possibly an amino acid residue, to yield a radical which is in magnetic dipolar contact with a strongly relaxing species such as a transition-metal ion or cluster. We believe the most reasonable candidate for the latter is the Mn cluster. These conclusions are in agreement with prior proposals based on more limited results (Boussac et al., 1989; Sivaraja et al., 1989b). If this interpretation is correct we can use the dipolar interaction to predict the distance of separation between the paramagnetic centers using the standard formula: $\partial H = (\mu_1\mu_2/R^3)(3\cos^2\phi - 1)$, $\mu = g\beta_e S$, etc. The 160-G line width can be attributed to a distribution of static dipolar fields at the radical site produced by the Mn cluster. The magnetic moment of the partner responsible for this broadening is unknown. Our magnetic susceptibility results support the claim that the magnetic moment of the Mn cluster has not changed appreciably in the S_3' and S_4' states from that observed in the modified S_2' state (Figure 8). If we take it to be the same as that for the ground state of the Mn cluster in the modified S_2' state, namely, total spin $S = {}^1/_2$ and $g = 2.0$, we obtain the distance of closest approach of 7 Å. If the spin of the partner is $S = 1$, then this distance would increase by 26% to 8.8 Å. We take this as the upper limit to the distance of separation.

Model for the Oxidation Steps in Ca-Depleted PSII. A model is given in Scheme I which summarizes our interpretation of the oxidation events which occur in Ca-depleted PSII. We start in the S_2' oxidation state with the Mn cluster in the mixed-valence state and two neighboring amino acid residues, R_1 and R_2. The S_2' state produces the modified multiline EPR signal. The oxidation state of the Mn ions has not been established in calcium-depleted samples. However, the EPR results clearly indicate a mixed-valence cluster with $S = {}^1/_2$ ground state analogous to the normal S_2 state. This has been shown to be most consistent with Mn(III)3Mn(IV) (Dismukes et al., 1981, 1982; Miller & Brudvig, 1989). On the first

turnover, R_1 is oxidized by one electron to the radical R_1^+ which is strongly coupled to the Mn cluster, resulting in the disappearance of the modified multiline EPR signal observed in the S_2' state. This state is EPR-silent. The second turnover creates S_4' by oxidizing another amino acid residue, R_2, yielding the radical R_2^+ which is weakly coupled to the Mn cluster and producing the dipolar-broadened 160-G-wide EPR signal. This model predicts one EPR-active radical per PSII, R_2^+, in agreement with the one to two spins observed experimentally. In this model, R_1 could be the hidden reductant whose oxidation is hypothesized to be responsible for loss of the S_2' multiline EPR signal. The same species could account for the reduction of Tyr-D$^+$ upon Ca^{2+} reconstitution (Sivaraja et al., 1989b; Lockett et al., 1990). Inclusion of a hidden reductant also helps to explain the variability seen in the flash yield of the 160-G-wide EPR signal.

A second interpretation of the same scheme is possible if we take the upper limit of two spins per PSII for the yield of the 160-G-wide signal. In this case, both R_1^+ and R_2^+ could give rise to this signal, and their coupling to the Mn cluster could be responsible for loss of the S_2' multiline EPR signal from broadening.

Photoinhibition. In Figure 1, we found that the yield of the 160-G-wide signal reaches a maximum and then diminishes with continued illumination. The kinetics depend on Chl concentration and light intensity. In Figure 6, we found that with increasing preillumination time calcium-depleted PSII membranes undergo irreversible damage due to multiple turnovers, resulting in the loss of recovery of O_2 evolution upon subsequent Ca reconstitution. At present, we are unable to quantitatively compare the yield of the 160-G-wide signal and the extent of Ca-recoverable O_2 rate activity, because these experiments must be performed at greatly different chlorophyll concentrations (5 mg/mL vs 40 μg/mL, respectively). Boussac et al. (1989) have shown that the state responsible for the 160-G-wide signal decays back to a normal S_1 state via the normal S_2 state following calcium readdition. Therefore, it can be reactivated. These observations indicate that the state yielding the 160-G-wide signal S_3' (Boussac et al., 1989) or S_3' or S_4' (this work) may be a precursor to photoinhibition and that attempted turnover beyond this state leads to irreversible loss of O_2 evolution capacity.

Normal vs Aberrant Photochemistry. Is it possible that the photooxidation reaction which yields the new radical signal in Ca-depeted PSII may occur in the native water-oxidizing complex, or is it merely a nonphysiological reaction? There are a number of observations reported in the literature which can be explained if oxidation of a component other than the Mn cluster occurs as part of the normal photochemistry. The absence of a radical EPR signal during normal cycling of the water-oxidizing complex could be explained if there is strong magnetic coupling between the radical and the Mn cluster. This view is consistent with the work by Guiles et al. (1990), who found from Mn X-ray absorption studies that a partially occupied S_3 state has the same K-electron edge energy as does S_2. Also, Dismukes and Mathis (1984) reported on near-infrared absorption changes in PSII membranes which were attributed to oxidation-state changes of manganese in the water-oxidizing complex. We found that the absorption spectrum and molar absorptivity were the same in the S_2 and S_3 states, suggesting that something other than manganese might be oxidized during this transition. One possibility is that photooxidation of a ligand to the Mn cluster could occur on the $S_2 \rightarrow S_3$ transition. However, more compelling evidence is needed to be certain.

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Registry No. Ca, 7440-70-2; Mn, 7439-96-5; Tyr, 60-18-4.

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A Phosphatase Inhibitor Enhances the DNase I Sensitivity of Active Chromatin†

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ABSTRACT: Although it is well-known that active domains of chromatin have elevated DNase I sensitivity, it can be difficult to observe preferential sensitivity in many cell types. We show that the DNase I sensitivity of active chromatin is enhanced some 10-fold by treating nuclei with the phosphatase inhibitor *p*-(chloromercuri)benzenesulfonic acid (CMBS) whereas DNase I sensitivity in inactive domains is only 3-fold higher. We further show that CMBS-enhanced DNase I sensitivity is associated with at least two histone modifications. First, the negatively charged CMBS molecule becomes covalently attached to the thiol groups on histone H3. Second, histone H2A phosphorylation is significantly elevated in treated nuclei. The phosphorylation data along with other results point to the possibility that H2A phosphorylaation plays a role in enhancing preferential DNase I sensitivity. Whatever the mechanism, CMBS treatment of nuclei followed by DNase I digestion provides a novel and reproducible assay for probing the chromatin structure of active domains.

Pancreatic DNase I detects two structural features that are characteristic of active chromatin. First, there are the short 100–400 bp openings in chromatin, the DNase I hypersensitive sites (Gross & Garrard, 1988), which often indicate binding sites for transcription factors. The second structural characteristic of active chromatin is domain DNase I sensitivity (Weintraub & Groudine, 1976; Stalder et al., 1980; Wood & Felsenfeld, 1982), which extends over many kilobases of chromatin and encompasses nontranscribed as well as transcribed DNA sequences (lawson et al., 1982; Alevy et al., 1984; Jantzen et al., 1986). While little is known about the structural features responsible for domain DNase I sensitivity, it is thought to render a chromatin domain accessible to regulatory factors and may be an obligatory step in the initiation of RNA transcription (Weintraub, 1985).

While DNase I hypersensitivity is readily detectable in all eukaryotic cells, current procedures for detecting domain DNase I sensitivity work poorly, if at all, for many cell types. We reasoned that endogenous nucleases, proteases, or phosphatases may degrade nuclear preparations and dampen the differences between active and inactive domains. Therefore, we searched for a broad-based inhibitor of degradative enzymes which would allow the reproducible detection of domain DNase I sensitivity in all cell types.

Several sulfhydryl reagents have been reported to inhibit dephosphorylation and proteolysis of histones (Paulson, 1980). In this report, we show that the irreversible sulfhydryl reagent *p*-(chloromercuri)benzenesulfonic acid (CMBS)[1] induces a dramatic increase in DNase I sensitivity in active chromatin, whereas inactive chromatin is less affected. We also explore the possible mechanisms of CMBS-enhanced DNase I sensitivity.

Experimental Procedures

Recombinant DNA. The full-length RB clone [p4.7R cDNA described in Friend et al. (1986)] was obtained from the Massachusetts Eye and Ear Infirmary. Plasmids containing the β-actin and AS coding regions (Beaudet et al., 1986) were obtained from Svend Freytag.

Cell Culture, Nuclear Isolation, and DNase I Digestion. RPMI 2650 cells (transformed nasal septum cells, ATCC CCL 30), Wilm's tumor cells (ATCC CRL 1441), and ductal

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[1] Abbreviations: CMBS (CMPS in figures), *p*-(chloromercuri)-benzenesulfonic acid; IAA, iodoacetamide; DEPC, diethyl pyrocarbonate; AS, argininosuccinate synthetase; RB, retinoblastoma; RSB, nuclear isolation buffer (10 mM Tris-HCl, 10 mM NaCl, and 3 mM $MgCl_2$); kb, kilobase(s).

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