Calcium Limits Substrate Accessibility or Reactivity at the Manganese Cluster in Photosynthetic Water Oxidation†

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ABSTRACT: The structural changes which the tetramanganese cluster responsible for catalyzing photosynthetic water oxidation undergoes upon calcium depletion of photosystem II membranes via the citrate extraction method has been further characterized. The modified multiline EPR signal which forms has been further identified with an S_2' oxidation state. The increased number of hyperfine lines (at least 26) and 25% narrower hyperfine splittings from ^{55}Mn versus the normal S_2-state signal indicate a redistribution of spin density, most likely within a spin-coupled tetranuclear Mn cluster. A simpler binuclear Mn description for this signal can be eliminated. Slow conformational changes occur over 30 min which cause subtle changes in the hyperfine structure. Comparison to the modified multiline signals produced by Sr^{2+} replacement of Ca^{2+} and NH_3-treated PSII reveal similarities suggestive of formtion of the same spin $S = {}^1/_2$ state. Substrate accessibility in the dark S_1' state, measured as Mn^{2+} release upon incubation with NH_2OH, is increased by 10-fold over calcium-containing PSII centers. Diphenylcarbazide, an efficient electron donor to Tyr-Z^+ only in PSII membranes in which Mn is removed or dislocated, was found to donate electrons in Ca^{2+}-depleted PSII, indicating altered accessibility or reactivity. These results suggest a possible "gatekeeper" role for Ca^{2+} in *limiting* access of substrate water to the Mn cluster. These changes are not due to release of the three extrinsic polypeptides of PSII which remain bound. Citrate treatment also causes partial air oxidation of the reaction-center Fe^{2+} ion, associated with the quinone electron acceptors. The resulting Fe^{3+} possesses an EPR signal at $g = 4.37$ arising from conversion to a rhombic symmetry ligand field.

Calcium is an essential cofactor for photosynthetic O_2 evolution in higher plants (Brand & Becker, 1984; Barr et al., 1980; Ono & Inoue, 1983; Tamura & Cheniae, 1987) and for photosystem II (PSII) electron transport in cyanobacteria (Piccioni & Mauzerall, 1976; Brand et al., 1983; Satoh & Katoh, 1985; Kashino et al., 1986). However, its function and site of action in water oxidation have not been established.

Evidence for the rebinding of Ca^{2+} to denatured PSII polypeptides visualized by emission from ^{45}Ca has implicated two proteins: the light-harvesting complex (LHC)[1] antenna complex and an unidentified polypeptide of mass ca. 33 kDa (Webber & Gray, 1989). On the basis of the apparent molecular weight, the latter site could be the extrinsic manganese-stabilizing protein (MSP) or the D_1 or D_2 reaction-center subunits. Using Ruthenium Red, which is an inhibitor of Ca^{2+} transport across chloroplast membranes, Lemieux et al. (1987) found that the Ca^{2+} binding site is not on the exposed surfaces of extrinsic polypeptides but lies closer to the reaction center. Coleman and Govindjee (1987) proposed the existence of a metal binding site on the 33-kDa MSP based on sequence analysis. Hunziker et al. (1987) have measured a dissociation constant of 1 mM for calcium binding to the isolated MSP at a site which also binds Mn^{2+} at a substoichiometric level (K_D = 2.6 mM, 0.14 Mn/MSP). Although this dissociation constant is in good agreement with the loosely bound calcium pool in PSII, the low stoichiometry and affinity suggests this involves nonspecific binding.

On the basis of sequence analysis of four gene products, Dismukes (1988) has identified 2 sites on the D_1 protein which possess an amino acid sequence analogous to that typical of so-called "E-F hand" calcium binding sites observed in 12 crystallographically determined Ca^{2+} binding proteins. One of the two sites bears a stronger homology. A similar proposal has been suggested for the MSP, although the sequence homology is considerably weaker (Burnap et al., 1990).

Recently three groups discovered that inhibition of O_2 evolution by extraction of Ca^{2+} from PSII membranes imparts a structural change of the manganese cluster as seen by formation of a new modified multiline EPR signal (MML), attributed to an S_2 oxidation state of the water-oxidizing complex. This structural change causes enhance kinetic and thermodynamic stability of the S_2 state of the tetramanganese cluster. It can be generated either by Ca^{2+} extraction with citric acid at pH 3 (Sivaraja et al., 1989; Ono & Inoue, 1990; Baumgarten et al., 1990) or by NaCl/EDTA washing (the depleted 17- and 23-kDa extrinsic proteins had to be reconstituted for observation of the modified multiline signal) (Boussac et al., 1989, 1990).

In this paper, we report on a slow conformational change of the Ca^{2+}-depleted Mn cluster and identify a possible physiological role for Ca^{2+} in restricting access or reactivity of substrate at the Mn cluster. We show that the new structure of the Mn cluster produced by Ca^{2+} depletion is analogous to that previously reported for Sr^{2+} substitution and for NH_3 treatment, as deduced from the modified multiline EPR spectra. We find that citrate also modifies the acceptor side Fe^{2+} site by imposing coordination changes which induce partial air oxidation of Fe^{3+}.

Materials and Methods

PSII(BBY) membranes were prepared according to the method of Berthold et al. (1981) at pH 6.5 and stored at −80 °C in 0.4 M sucrose, 50 mM MES, 25 mM NaCl, and 15 mM

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[1] Abbreviations: DCBQ, 2,5-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; LDS-PAGE, lithium dodecyl sulfate–polyacrylamide gel electrophoresis; LHC, light-harvesting complex.

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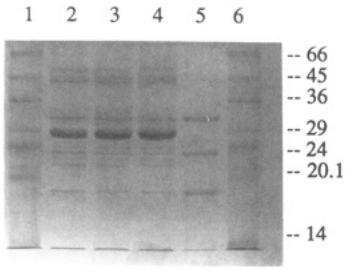

FIGURE 1: LDS–PAGE of (2) control PSII membranes, (3) Ca-depleted PSII membranes, (4) Ca-reconstituted PSII membranes, and (5) PSII extrinsic proteins of 33, 23, and 17 kDa. Lanes 1 and 6 are molecular weight standards. Gel conditions: 6 M urea, 15% acrylamide.

$CaCl_2$ until further use. Calcium-depleted PSII membranes were prepared according to the procedure outlined by Ono and Inoue (1988), involving extraction with 10 mM citric acid at pH 3.0 for 30 min at 277 K. Prior to centrifugation, the pH was adjusted to 6.5 with Ca-free buffer (0.4 M sucrose, 50 mM MES, and 25 mM NaCl, pH 6.5). A slight modification in the procedure involving reconstitution with divalent metals involved centrifugation of the diluted membranes in Ca-free buffer, followed by resuspension in the buffer containing the divalent metal salt to be reconstituted (50 mM $CaCl_2$ or $CdCl_2$, 0.4 M sucrose, 10 mM NaCl, and 10 mM MES, pH 6.5). Subsequent oxygen activity and Hill reaction measurements were then made with DCBQ and DCIP, respectively, as the exogenous electron acceptors. ESR measurements were performed as previously described (Sivaraja et al., 1989). Samples contained 1–2 mM DCBQ. LDS–PAGE was performed according to Laemmli (1970), with 6 M urea and 15% acrylamide.

Results

O_2 Evolution, Electron-Transfer Rate, and Protein Content of Ca-Depleted PSII Membranes. After treatment with citrate at pH 3.0, PSII membranes had O_2 evolution rates that were typically 15–20% of the untreated PSII membranes. The electron-transfer rate through the reaction center from the added donor DPC to the acceptor DCIP was typically 70% of the untreated membranes, in agreement with the results of Ono and Inoue (1988). Incubation of the citrate-treated samples with 50 mM $CdCl_2$ or 200 mM NaCl did not restore O_2 evolution activity, confirming the specifity for Ca^{2+} in these membranes. A stoichiometry of one Ca removed per PSII membrane was reported by Ono and Inoue (1988) and confirmed by Shen and Katoh (1990).

From reciprocal plots of the extent of reactivation of the O_2 evolution rate by added $CaCl_2$, we observed a biphasic dependence with dissociation constants of 80 μM and 6 mM. These are in close correspondence with the values found for the "intermediate"- and "low"-affinity dissociation constants reported for the 1 equiv of Ca^{2+} per PSII linked to reversible loss of O_2 evolution, 50–100 μM (Boussac et al., 1985; Cammarata & Cheniae, 1987) and 1–10 mM (Shen & Katoh, 1990). A higher affinity site of unclear stoichiometry has also been reported in the range 1–4 μM (Waggoner et al., 1990; Kalosaka et al., 1990).

Because loss of the extrinsic PSII polypeptides by salt washing greatly attenuates O_2 evolution activity (Miyao & Murata, 1984; Ghanotakis et al., 1984; Ono & Inoue, 1984; Ghanotakis & Yocum, 1985), we sought to determine if the inhibition by citrate might also release these proteins. Figure 1 depicts an LDS–PAGE slab where lanes 1 and 6 are molecular weight markers, lane 2 is an untreated PSII membrane sample, lane 3 is a citrate-treated sample, lane 4 is a citrate-treated sample reconstituted with 50 mM $CaCl_2$, and lane 5 shows the three extrinsic PSII polypeptides (33, 23, and 17 kDa) released by washing PSII membranes in 1 M $CaCl_2$. This comparison clearly shows that the citrate treatment does not release any of the PSII polypeptides when care is taken to adjust the pH from 3 to 6.5 following citrate incubation. Without this precaution, loss of these proteins can occur.

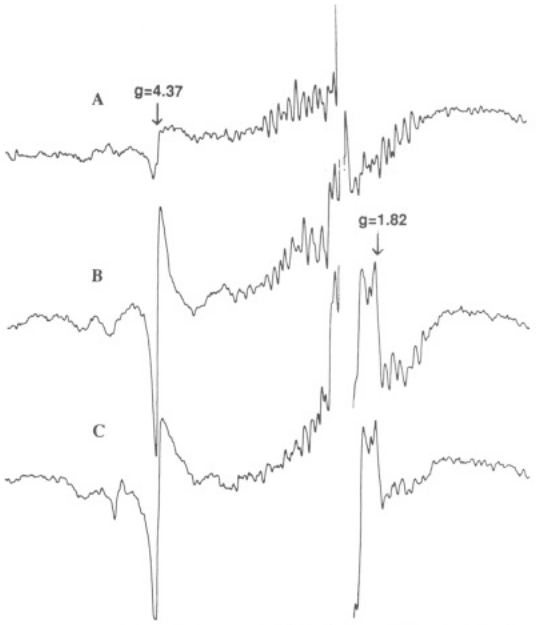

FIGURE 2: Formation of the modified S_2' multiline EPR signal in calcium-depleted PSII membranes (A) by 273 K illumination (20 s) followed by dark-adaptation (15 min) in the presence of the electron acceptor DCBQ or (B) by 273 K illumination (20 s) and immediate freezing to 77 K in a sample treated with the inhibitor DCMU. Curve C corresponds to a second turnover of the sample from curve A (see text). The spectra are presented as difference vs a dark-untreated PSII sample. DCBQ concentration = 0.5 mM, DCMU concentration for curve B = 150 μM and for curve C = 400 μM. T = 8 K, microwave power = 32 mW, modulation amplitude = 20 G.

By the criteria of O_2 evolution rate, electron-transfer rate, and polypeptide content, our samples behave the same as those described by Ono and Inoue (1989a) and Shen and Katoh (1990), who found additionally that the citrate treatment releases one of the two Ca^{2+} ions in PSII.

Multiple Turnovers. In a previous report, we showed that illumination at 273 K of rigorously dark-prepared Ca-depleted samples treated with the Q_B inhibitor DCMU, to restrict PSII to a single turnover, gives rise to a modified S_2' multiline EPR signal (Sivaraja et al., 1989). If the number of turnovers is not restricted by including an exogenous electron acceptor like DCBQ, it was reported that a new signal can be observed in its place at g = 2.004 with a symmetric line width of 160 G (Sivaraja et al., 1989; Boussac et al., 1989). These reports showed that dark re-adaptation results in loss of this signal along with recovery of the modified S_2' multiline signal. Figure 2A,B compares EPR difference spectra of the modified multiline signal generated by both means, using DCBQ or DCMU, respectively. The yield of this signal is comparable in both samples, demonstrating that a single turnover from the dark S_1' state produces the maximum yield of the modified multiline signal. By contrast, the yield of the 160 G wide signal is greatly depressed in the presence of DCMU (10%, curve B). What little forms presumably results from those centers which fail to bind DCMU. Ca-depleted samples containing DCMU also exhibit a strong light-induced Q_A^-Fe EPR signal at g 1.82 (curve B), comparable in yield to that of a calci-

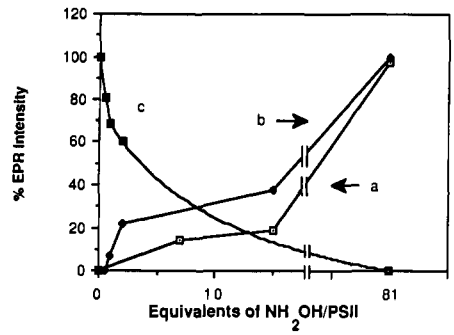

FIGURE 3: Release of Mn^{2+} by NH_2OH as seen by EPR from untreated (a) and calcium-depleted (b) PSII membranes. Curve c is a plot of the yield of the 160-G-wide $g = 2.0$ EPR signal. Other conditions as in Figure 2.

um-sufficient sample treated with DCMU (data not shown).

A new signal (Figure 2B) can be observed at $g = 4.37$ which is reversed phase, indicating that photoreduction of this species occurs even in the presence of DCMU. In curve A, the $g = 4.37$ signal is absent because the dark level of the signal is restored by the 15-min dark-adaptation procedure. The association of this signal with an oxidized form of the acceptor-side Fe^{2+} in the PSII reaction center is discussed in a later section.

A double-turnover experiment was performed to learn if a second turnover could generate the 160-G-wide signal. The sample from trace A has effectively undergone one net turnover from S_1' to S_2', and the acceptor side is re-oxidized during the dark re-adaptation period, as seen by the lack of the Q_A^-Fe EPR signal at $g = 1.82$ or 1.9 (Figure 2A). Subsequent addition of excess DCMU (400 μM) to displace the exogenous DCBQ from the Q_B site, followed by reillumination at 273 K, should allow a second turnover to occur, as has been widely observed in normal PSII membranes. Curve 2C shows the result of such an experiment. Confirmation that DCMU bound to the Q_B site is seen by the large $g = 1.82$ signal (about 70% of a single turnover, curve 2B). The second turnover causes the 160-G-wide signal to increase from 10% (single turnover) to 25% of the maximum yield, while the modified S_2' multiline signal decreases by greater than 50%. This shows that in the 70% centers which bind DCMU, a second turnover does not generate the 160-G-wide signal (the 25% yield of this signal we presume results from the 30% centers which did not bind DCMU). The greater than 50% loss of the modified S_2' multiline signal can be attributed to advancement to an S_3' state which is EPR-silent, analogous to the normal S_3 state. The 160-G-wide signal appears to arise only after more than two turnovers.

Release of Mn by NH_2OH in Ca-Depleted PSII Membranes. The reaction of bound Mn in the water-oxidizing complex with NH_2OH and its release as Mn^{2+} were compared in Ca-sufficient and Ca-depleted PSII membranes by monitoring the yield of the free Mn^{2+} EPR signal. The total amount of functional manganese extractable by NH_2OH is known to be 3–4/PSII depending on the conditions (Cheniae & Martin, 1971; Sivaraja & Dismukes, 1988). Figure 3 shows that at an excess molar ratio of NH_2OH per PSII centers (81 NH_2OH/PSII, 2 mM NH_2OH), a maximum of 3 Mn/PSII are released in both samples, under these conditions (Sivaraja & Dismukes, 1988). At lower NH_2OH concentrations, where partial Mn release occurs in the range 2–15 NH_2OH/PSII, it is clear that prior removal of Ca renders PSII more susceptible to Mn reduction and release; 20% of the Mn can be released by as few as 1.5 NH_2OH/PSII in Ca-depleted membranes, whereas a 10-fold greater concentration, about

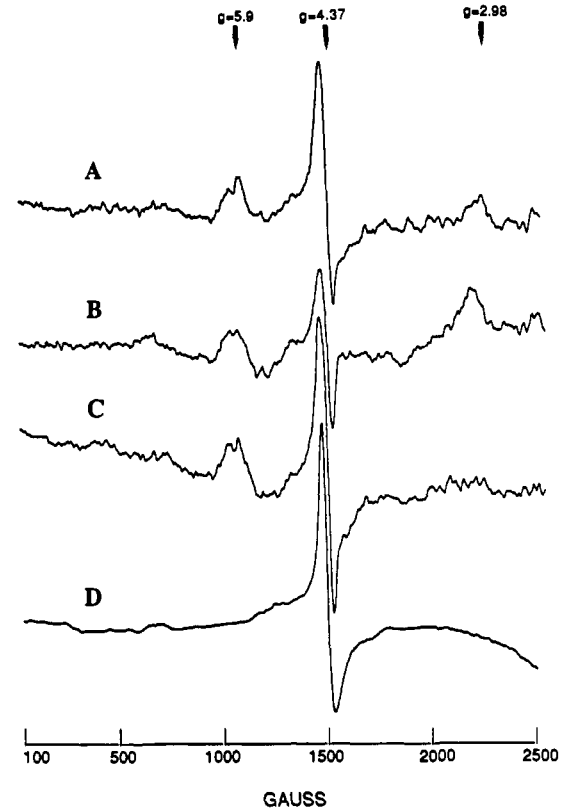

FIGURE 4: EPR difference spectra of the $g = 4.0$ region of (A) dark, (B) 200 K illuminated, and (C) dark re-adapted (15 min) calcium-depleted PSII membranes. Dark incubations are at 273 K. The difference is against a dark-untreated PSII sample. Spectrum D is a trace of 20 μM $FeCl_3$ in the 10 mM citrate buffer (pH 3.0) used for calcium depletion of PSII membranes. Other conditions same as for Figure 2.

15 NH_2OH/PSII, is required to release the same amount from untreated PSII membranes.

We also found that addition of 2 mM DPC 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^+.

Single-Turnover Photoreactions at 200 K. As previously shown, illumination of Ca^{2+}-depleted PSII membranes at 200 K fails to produce the S_2 multiline signal (dePaula et al., 1986a). We found this is also true of the $g = 4.1$ S_2 signal (not shown). Both signals form in untreated PSII membranes, the latter as a minor component. A small amount of cyt *b*-559 (20%) becomes photooxidized in the calcium-depleted samples as could be seen by the g_x peak at 3.0.

Identity of the $g = 4.37$ EPR Signal with the Acceptor-Side Fe(III). Figure 4 shows the $g = 4$ region of Ca-depleted PSII samples in the dark (A), after 200 K illumination (B), and after 15-min dark-re-adaptation (C). Curve D corresponds to a 20 μM solution of $FeCl_3$ in 10 mM citrate buffer, pH 3.0. Except for trace D, all the other spectra are presented as light minus dark difference spectra in which the dark-adapted sample is an untreated PSII sample. This removes the dark 120-G-wide "rhombic" $g = 4.3$ signal arising from nonspecific Fe(III). The g value of the new signal at 4.37, its 70-G peak-to-peak line width, and its microwave power dependence (data not shown) are very similar to those for $Fe(citrate)^{3+}$. From this, we conclude that the $g = 4.37$ signal arises from a ferric ion in a homogeneous ligand environment of rhombic symmetry similar to citrate coordination. A comparison of

the amplitudes of the signals in (A) and (D) shows that the signal in PSII (A) corresponds to a spin concentration of about 2.5 μM, which is less than 10% of the reaction-center concentration used in (A) (5 mg/mL Chl). Illumination of the sample in (A) at 200 K results in about 50% loss of the g = 4.37 signal (curve B), while subsequent dark-adaptation at 273 K for 15 min (curve C) restores the intensity of this signal to about its original level. Clearly, this indicates it originates from a reversibly oxidizable species located in the reaction center. Re-addition of $CaCl_2$ in the dark to the Ca^{2+}-depleted membranes restores their ability to photogenerate the normal S_2-state EPR signals, while abolishing both cyt b-559 photooxidation and the dark g = 4.37 signal in a majority of the centers (data not shown). An obvious candidate for this signal is the oxidized non-heme ferrous ion associated with the primary and secondary quinone electron carriers. Autooxidation to Fe(III) has been found with other reagents which have access to the Q_B site (Petrouleas & Diner, 1986; Zimmerman & Rutherford, 1986). The smaller signal appearing at g = 5.9 in the spectra of Figure 4 is unaffected by illumination, or by re-addition of Ca^{2+}. Accordingly, it must originate from a different spin system than the g = 4.37 signal.

Discussion

Characteristics of the Ca^{2+} Site. It is important to establish the characteristics of the PSII samples following citrate treatment in order to judge whether the effects we observe can be ascribed solely to the selective removal of calcium. Our data on the recovery of O_2 evolution by Ca^{2+} reconstitution (not shown) yielded two dissociation constants of 80 μM and 6 mM. These fall in the middle of each of the ranges reported in the literature for the two lower affinity sites, as we noted above. The citrate treatment has been shown by two groups to remove only a single Ca^{2+} ion. The existence of two binding affinities for 1 equiv of calcium appears to reflect heterogeneity in the PSII membranes. This heterogeneity has been further characterized by Shen and Katoh (1990), who observed that NaCl washing of low-pH/citrate-extracted PSII membranes induced a greater fraction of the higher affinity rebinding. They attributed this to release of the 23- and 17-kDa proteins by salt treatment. No detectable protein release occurred in our samples using the citrate extraction method, as seen by protein electrophoresis (Figure 1).

Substrate access to the Mn cluster is enhanced in Ca-depleted PSII membranes. The above data demonstrate that the 10-fold-enhanced release of Mn by NH_2OH in citrate-treated samples, noted in Figure 3, is not due to release of the extrinsic proteins. It presumably reflects a direct consequence of the Ca^{2+} removal. Binding of calcium to the site which is coupled to water oxidation appears to render the Mn cluster either less accessible to small molecules such as NH_2OH and H_2O in the S_2' state (kinetic control), or a weaker oxidant for NH_2OH (thermodynamic control). The former explanation was also the conclusion of our earlier work in which greater access of water to the Mn site was invoked to explain the enhanced stability of the S_2' state via spontaneous disproportionation of the modified Mn cluster, by analogy to the chemistry observed for organic-soluble binuclear Mn complexes upon equilibration with water (Sivaraja et al., 1989). This explanation also allows for a thermodynamic origin for the difference in Mn release. For example, the disproportionation reaction in model complexes, $2Mn_2(III,III) \rightarrow Mn_2(II,III) + Mn_2(III,IV)$, becomes thermodynamically spontaneous only in the presence of water, owing to formation of the stable bis(μ-oxo) ligand bridge between Mn(III) and Mn(IV). Assignment of the enhanced S_2' stability in Ca-depleted PSII to a lowering of the oxidation potential for the $S_1' \rightarrow S_2'$ reaction has been the interpretation advanced to explain the greatly elevated activation temperature for induction of thermoluminescence via $S_2'Q_A^-$ recombination (Ono & Inoue, 1989b).

Partial prereduction of the NH_2OH-releasable Mn by Ca depletion without changing its reduction potential cannot account for the data of Figure 3. It has been previously established that reductive release of manganese is a cooperative process, about 3 Mn/PSII, for which a single step controls Mn release (Sivaraja & Dismukes, 1988; Sivaraja et al., 1988).

There is discussion that the NH_2OH binding site may not correspond to the substrate binding site, but rather to the Cl^- binding site required for O_2 evolution (Beck & Brudvig, 1988). This is discussed by Tso et al. (1990), who failed to observe competition between Cl^- and the analogous inhibitor *N,N*-dimethylhydrazone, $(CH_3)_2NNH_2$, and therefore concluded that this inhibitor does not bind to the Cl^- site. The Cl^- suppression of the rate of inhibition by *N,N*-dimethylhydroxylamine monitored by Beck and Brudvig in the range 20–50 mM NaCl occurs at 100-fold higher Cl^- concentration than that associated with stimulation of O_2 evolution. The effect thus appears to represent a nonspecific electrostatic screening which slows the rate of inhibitor binding, but does not compete with it directly.

The observation that the bulky electron donor, DPC, can compete for electron donation to Tyr-Z^+ in Ca^{2+}-depleted PSII membranes, but not in normal PSII, also suggests that a structural change occurs upon Ca^{2+} depletion which increases accessibility to Tyr-Z^+.

The present results further support our original model in which calcium serves as a "gatekeeper" to limit access of water and substrate analogues to the manganese cluster in its lower oxidation states (Sivaraja et al., 1989). We further hypothesize that once exposure to water is permitted, then a thermodynamically favorable disproportionation reaction may take place which is common to synthetic Mn clusters (Sivaraja et al., 1989). It appears that too much water is not a good thing for the water-oxidizing complex.

Single Turnover at 200 K. Illumination at 200 K of citrate-treated PSII membranes fails to generate a normal S_2 state as seen by the loss of the S_2-state EPR signals, both the multiline and the g = 4.1 signals. Restoration of the multiline signal and some of the g = 4.1 signal occurs with addition of $CaCl_2$ to a level commensurate with the restored O_2 evolution rate. This confirms prior studies showing that Ca depletion by 200 mM NaCl washing eliminates the multiline signal in a reversible manner (Miller et al., 1987). However, contrary to this report which attributes this to a block at the $S_1 \rightarrow S_2$ reaction, we observe no evidence for a block in this transition. This was also the conclusion reached by Ono and Inoue (1989b) using thermoluminescence. Turnover to form the normal acceptor-side EPR signals at g = 1.9 and g = 1.82 EPR signals FeQ_A^- occurs under these conditions. We are uncertain as to the identity of the major electron donor responsible for Q_A photoreduction [cyt b-559 at g = 3.0 is a minor donor (20%) at this illumination temperature]. It is possible that the Mn cluster is still photooxidized but is structurally modified to yield an EPR-silent state. A similar phenomenon is observed in chloride-depleted PSII membranes, where illumination results in Mn oxidation, but the resulting S_2' state has greatly reduced EPR intensity (Casey & Sauer, 1984; Damoder et al., 1986). Re-addition of chloride to this modified state regenerates the normal S_2 state, which is EPR-active (Ono et al., 1986). Both the calcium-depleted and chloride-depleted

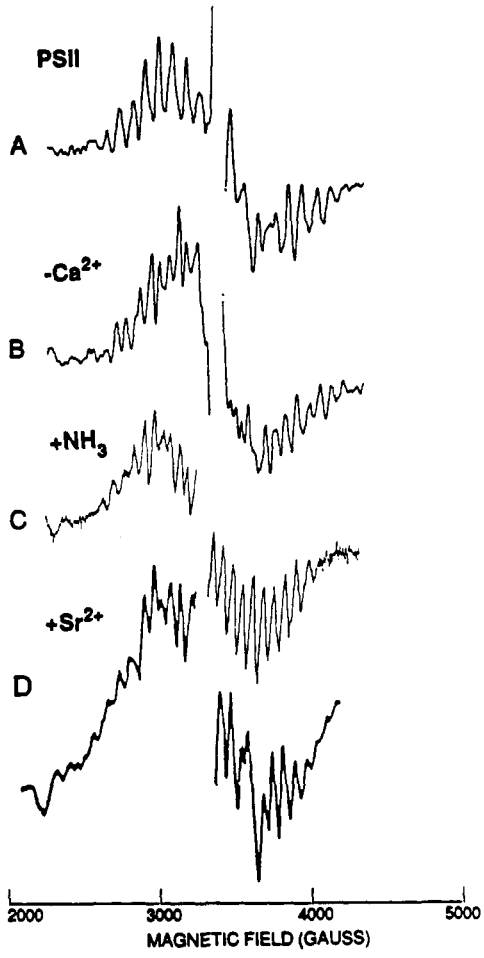

FIGURE 5: Comparison of the multiline EPR signal in (A) untreated (S_2), (B) Ca^{2+}-depleted (S_2'), (C) NH_3-treated [$NH_3(S_2')$], and (D) Ca^{2+}-depleted and then Sr^{2+}-reconstituted [$Sr(S_2')$].

PSII preparations share an abnormally stable S_2' state as seen by an upshifted temperature for thermoluminescence (Vass et al., 1987; Ono & Inoue, 1989).

Modified S_2 Multiline EPR Signal. The comparable yields of the modified S_2' multiline signal observed in samples illuminated in the presence of DCMU to restrict turnover to one electron (Figure 2B), versus dark-adaptation of a sample after multiple turnovers (Figure 2A), clearly demonstrate that the modified multiline signal arises from an S_2' state which is 1 oxidizing equiv above the dark (S_1') state. We and others previously attributed this to an S_2' state on the basis of other results (Boussac et al., 1989; Sivaraja et al., 1989; Ono & Inoue, 1990).

In Figure 5, we compare the multiline signals arising from the normal S_2 state, the S_2' state of citrate-treated PSII membranes, the $S_2'(Sr)$ state of Ca-depleted membranes reconstituted with Sr^{2+} (Boussac & Rutherford, 1988), and the S_2' (NH_3) state of ammonia-treated PSII membranes (Beck et al., 1986). The overall width of the hyperfine field in all cases is about the same (1500 G). The most striking difference between the normal S_2 multiline spectrum in trace A versus all of the others is the number of lines, 17–18 versus 24–25, respectively, and the hyperfine splittings between them, 80–90 G versus 65–70 G.

The normal S_2 multiline EPR signal has been assigned to a spin $^1/_2$ ground state of an antiferromagnetically coupled cluster of four Mn ions (Dismukes & Siderer, 1981; Dismukes et al., 1982, Dismukes, 1986; dePaula et al., 1986b). The very similar hyperfine characteristics for the modified multiline spectra in Figure 5B–D indicate that a single new spin state forms in all three cases. The observed hyperfine splittings for ^{55}Mn in a tetranuclear cluster which is coupled by spin exchange can adopt only selected values. It is a function of the spin density on the individual ions which in turn is a function of the covalency of the ligand–Mn bonds, as it would be in a monomer, but is also multiplied by the projection of each ion's spin onto the total cluster spin (S_iS_T/S_T^2). The latter occurs because the hyperfine interaction involves coupling between each nucleus and the electron spin of that atom, while the electron spin of each atom is strongly coupled to the other paramagnetic atoms in the cluster to yield a resultant cluster ground state of $S_T = {}^1/_2$. This relationship has been well documented for Mn dimers (Dismukes et al., 1982, 1987; Dismukes, 1990). Because there is only a single spin $^1/_2$ state for mixed-valence Mn_2(III,IV) complexes, it is not possible to account for the spectral changes which are observed for the Mn cluster using a simple dimer description of the Mn cluster.

There are eight nondegenerate $S_T = {}^1/_2$ doublet states in an exchange-coupled tetranuclear cluster of symmetry lower than cubic comprised of three Mn(III) and one Mn(IV) (Dismukes et al, 1982). Each spin $^1/_2$ state has a different set of hyperfine constants for Mn. The decrease in the observed hyperfine splitting without a decrease in the total hyperfine field width observed in curves B, C, and D cannot be due principally to an increase in the Mn–ligand covalency, since this would reduce the width of the hyperfine field arising from Mn. This argues in favor of the formation of a new cluster ground state resulting from stabilization of a different spin $^1/_2$ doublet of the tetrameric cluster. This could arise by changing the bridging ligand geometry or identity between the Mn ions. Spectral simulations are in progress to quantitatively test this hypothesis. From the temperature dependence of the $S_2'(NH_3)$ multiline EPR signal, de Paula et al. (1986a) invoke this same explanation to account for the reduction in the observed hyperfine splitting in the $S_2'(NH_3)$ multiline signal. From electron spin-echo studies of the multiline signal, Britt et al. (1989) have shown that NH_3 binds in very close proximity to the Mn cluster, probably as a direct ligand to manganese. From EXAFS studies, Penner-Hann et al. (1990) have suggested tentatively that there may be a Ca site located 4.5 Å away from one of the Mn ions in the cluster. This distance is close enough for Ca to share a common amino acid ligand with the Mn cluster, and its release from the site could account for the structural modification of the Mn cluster upon Ca depletion seen by EPR. However, a more distant conformational coupling with the Ca site cannot be ruled out on the basis of our data. This includes the postulated calcium binding site located at the luminal surface of the membrane-spanning E helix of the D_1 polypeptide. This is adjacent to the 50 amino acid residues comprising the carboxyl terminus of D_1 which have been the focus of proposals for Mn binding sites.

Registry No. Ca, 7440-70-2; Mn, 7439-96-5; O_2, 7782-44-7; H_2O, 7732-18-5; Fe, 7439-89-6.

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