Intermediates of a polynuclear manganese center involved in photosynthetic oxidation of water

(electron paramagnetic resonance/manganese enzyme/oxygen evolution/electron transport)

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ABSTRACT Electron paramagnetic resonance of spinach chloroplasts given a series of laser flashes, $n = 0, 1, \dots, 6$, at room temperature and rapidly cooled to -140°C reveals a signal possessing at least 16 and possibly 21 or more hyperfine lines when observed below 35 K. The spectrum is consistent with a pair of antiferromagnetically coupled Mn ions, or possibly a tetramer of Mn ions, in which Mn(III) and Mn(IV) oxidation states are present. The intensity of this signal peaks on the first and fifth flashes, suggesting a cyclic change in oxidation state of period 4. The multiline signal produced on the first flash is not affected by the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea but is abolished by agents that influence the state of bound manganese, such as incubation with alkaline Tris, or dithionite, and by extraction with cholate detergent in the presence of ammonium sulfate. These results indicate that the paramagnetic signal is monitoring oxidation state changes in the enzyme involved in oxidation of water.

The oxidation of water to form molecular oxygen during the light reactions of photosynthesis in green plants and algae serves as the principal source of oxygen in the atmosphere. Although the light-induced reactions in photosynthetic organisms have been studied in great detail with much success, the species that binds two water molecules and extracts four electrons and four protons, by an as yet unknown mechanism, has not been identified. Recently, it was reported that oxygen evolution is reversibly lost from thylakoid membranes when a protein containing 2 Mn ions is removed with the aid of cholate detergent (1); 85% of the original oxygen yield could be restored upon reconstitution of this Mn protein into the depleted thylakoid membranes. The structure of this protein and the mechanism by which it operates are currently unknown. A widely accepted hypothesis based on the yield and kinetics of oxygen release in response to successive light flashes suggests that an enzyme, having five possible metastable redox states related by one-electron oxidations, couples chlorophyll photooxidation to water oxidation (2). There is considerable chemical and indirect spectroscopic evidence implicating manganese in the water-oxidation center, and an apparent stoichiometry of 4 Mn per enzyme has been estimated (reviewed in refs. 3 and 4).

A discussion of the energetics of the photosynthetic water-oxidation reaction has been presented and a hypothetical mechanism, based on little evidence, has been proposed in which binuclear Mn binds the oxidized intermediates H_2O_2 , O_2^- , and O_2 (5, 6). Flash-dependent changes of a six-line Mn electron paramagnetic resonance (EPR) signal observed at room temperature have been reported (7). Although a coupling to electron transport appears to be present, an association with the water-oxidation site could not be established. Indirect evidence for oxidation states higher than Mn(II) has been claimed based on flash-dependent ^1H NMR relaxation changes (8) and from

changes in the yield of soluble $\text{Mn}(\text{H}_2\text{O})_6^{2+}$ following flash excitation and heat shock (9).

We report direct EPR spectroscopic evidence for a binuclear or possibly tetranuclear Mn center in spinach chloroplasts. The intensity of the EPR signal of this center depends on the number of light flashes the sample receives in a manner which indicates sequential one-electron photooxidations (10). The effects of detergent fractionation and electron transport inhibitors suggest that the signal arises from the water-oxidation site of Photosystem II.

MATERIALS AND METHODS

Broken spinach chloroplasts were prepared as described (11). Rates of O_2 evolution, with $0.75 \text{ mM Fe}(\text{CN})_6^{-3}$ as electron acceptor, were typically $69 \mu\text{eq/hr}$ per mg of chlorophyll (Chl) compared to $402 \mu\text{eq/hr}$ per mg of Chl for intact (CO_2 -fixing) chloroplasts. The rate was increased 9- and 6-fold, respectively, upon addition of the uncoupler NH_4Cl at 5 mM . Chloroplast pellets were suspended in an equal volume of SHN medium (0.4 M sucrose 0.05 M Hepes, $\text{pH } 7.5/0.01 \text{ M}$ NaCl) and made 3 mM in EDTA. All chloroplast samples contained $25 \mu\text{g}$ of ferredoxin per ml and 1 mM NADP $^+$ (Sigma); 50% (vol/vol) glycerol was added to all samples after incubation with electron acceptors or inhibitors and prior to flashing. Final Chl concentrations were between 2 and 4 mg/ml . All samples were kept on ice prior to flashing. Some samples were incubated with 0.8 M Tris/ 0.01 M NaCl, $\text{pH } 8$, for 20 min prior to pelleting by centrifugation, after which EPR samples were prepared as above. Other samples were incubated with either 0.1 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or 0.05 M sodium dithionite. Extraction of thylakoid membranes with cholate was performed as reported (1). The cholate-extracted samples contained 0.5 mM diphenylcarbohydrazide and 1 mM methyl viologen as electron donor and acceptor, respectively.

EPR samples were contained in 4.4-mm outer diameter thin-walled Suprasil quartz tubes. These were transferred from ice to a holder positioned directly above an isopentane bath cooled to -140°C . Illumination was provided by a 25-nsec pulse of light at 532 nm produced by a Nd/YAG laser (Molelectron Corp). The 8-mm diameter light beam was expanded with a cylindrical lens to expose a 3-cm height of sample; a mirror directed the transmitted beam to the back of the sample. The light intensity was nominally 0.03 J/cm^2 . Light flashes were separated in time by $\approx 0.5 \text{ sec}$. Immediately following exposure, samples were plunged into the isopentane bath which provided rapid cooling. After 15 sec , samples were transferred to liquid N_2 prior to EPR measurement at or below 25 K . In some experiments, samples

Abbreviations: EPR, electron paramagnetic resonance; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Pcy $^+$, oxidized plastocyanin.

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FIG. 1. First-derivative EPR spectra of spinach chloroplasts flashed n times ($n = 0-6$) at room temperature prior to quench cooling to -140°C . Measurement temperature, 10 K; microwave power, 150 mW; microwave frequency, 9.45 GHz; modulation amplitude, 32 G; receiver gain, 500. (Inset) Modulation amplitude, 20 G.

that were flashed n times prior to freezing were thawed and flashed $n + 1$ times and frozen again. This was repeated until each sample had been flashed and thawed five times. In order to differentiate between flash-induced effects and freeze/thaw effects, other samples were frozen and thawed but not flashed for comparison.

EPR measurements were performed by using a Varian E-12 spectrometer at X-band and 100-kHz field modulation with temperature control provided by the off-gas from liquid helium (Helitran cryostat, Air Products, Inc.)

RESULTS

The EPR spectra of chloroplasts that were flashed n times ($n = 0, 1, 2, 3, 4, 5$, and 6) prior to quench cooling are shown in Fig. 1. A prominent multiline signal was produced in the $g = 2$ region on the first flash and decreased on the second flash. This signal appeared again with increased intensity on flash 5 and decreased again on flash 6. An expanded vertical scale of the spectrum observed after one flash shows that the apparent hyperfine structure stretches over more than 1400 G. At least 16 lines are clearly present with an average separation of 75–90 G. There appear to be additional weak hyperfine transitions in

FIG. 2. Dependence of average intensity of the three strongest hyperfine peaks from Fig. 1 on flash number.

the wings of the spectrum outside of the prominent 16 inner lines. The central transitions exhibited additional structure when observed at a lower modulation amplitude (Fig. 1 Inset). A graph of the intensity of the strongest hyperfine peaks (Fig. 2) reveals the magnitude of the signal intensity changes with flash number. Decreasing the temperature between 35 and 7.5

FIG. 3. Effect of electron transport inhibitors and cholate extraction on the multiline signal produced by a single flash and quench cooling. Spectrometer conditions were as in Fig. 1 except that the cholate data were recorded at modulation amplitude 25 G. The difference spectrum was obtained by digital subtraction using a Northern NS-575 signal averager.

K increased the intensity of the signal under nonsaturating microwave powers (data not shown).

Incubation of chloroplasts with 0.1 mM DCMU under ambient light appeared not to alter the yield of the signal produced by a single flash (Fig. 3). Chloroplasts incubated with Tris or with 0.05 M sodium dithionite showed no multiline signal. Thylakoid membranes that were extracted with cholate to remove a Mn-containing protein (1) did not show the same multiline signal observed in chloroplasts. These depleted membranes are capable of light-induced water oxidation only when the extracted Mn protein is added back (1).

In order to determine if the hyperfine transitions from oxidized plastocyanin (Pcy^+) might also be contributing to part of the hyperfine structure observed on the low-field side of $g = 2$, we continuously illuminated a sample containing 0.1 mM DCMU and quench cooled it to -140°C . The characteristic signal at $g = 2.05$ for Pcy^+ (not shown) was found to increase in intensity by at least 10-fold, indicating that for the single flash experiments it was largely reduced. Moreover, the Pcy^+ hyperfine structure near $g_{\parallel} = 2.24$ was poorly resolved in comparison to that in Fig. 1 and consisted of only four peaks having a separation of 60 G. These results show that Pcy^+ is not masking part of the low-field side of the multiline spectrum.

DISCUSSION

The reasons for the lack of success by previous researchers in observing this multiline signal seem to be the weak signal intensity caused by the extensive hyperfine structure, the temperature sensitivity of the signal, and the need to generate and trap a large population by a brief flash in a high optical density sample.

A difference spectrum between the EPR signals observed after one flash and no flash is shown in Fig. 4B. This is compared

FIG. 4. EPR spectra of the synthetic dimer

$(\text{bipy})_2\text{Mn(III)}-\text{O}-\text{Mn(IV)}(\text{bipy})_2$ (A), difference between the 1 and 0 flash spectra from Fig. 1 (B), computer simulation of B assuming a (III,IV) dimer with $|A_1| = 177$ G and $|A_2| = 82$ G and linewidth given in the text (C), stick spectrum corresponding to C (D), and stick spectrum for a (II,III) dimer with $|A_1| = 216$ G and $|A_2| = 113$ G (E).

to a spectrum for the synthetic mixed-valence Mn dimer

$(\text{bipy})_2\text{Mn(III)}-\text{O}-\text{Mn(IV)}(\text{bipy})_2$ (bipy = 2,2'-bipyridine) given

in Fig. 4A. The hyperfine structure for the synthetic dimer bears a great similarity to the prominent 16 interior lines observed in the chloroplast samples, thus implicating a pair of exchange coupled Mn ions.

The ions are known to be antiferromagnetically coupled in the synthetic dimer, having an exchange integral $J = -150 \pm 7 \text{ cm}^{-1}$ and exhibit inequivalent Mn hyperfine constants (12). An exchange integral $|2J| \geq 10 \text{ cm}^{-1}$ can be estimated from the temperature dependence of the chloroplast spectrum (not shown). The nearly isotropic hyperfine tensors of the Mn ions and the g tensor are exemplified by the well-resolved features in the spectrum of Fig. 4B, suggesting a total spin $S = 1/2$ for the center. The EPR properties can be explained in terms of a mixed-valence oxidation state in which the two Mn ions differ by one electron. This results in a ground state having $S = 1/2$ for an antiferromagnetically coupled complex. For the spectrum of Fig. 4B, $g = 1.96 \pm 0.02$ when corrected for the second order hyperfine shift of the spectrum. This is near the value $g = 2.003$ found for the synthetic dimer. The small g shift from the free electron value and the nearly isotropic character indicate that octahedral coordination and high spin ligand fields around the Mn ions are likely to exist.

All hyperfine transitions for the synthetic dimer have been assigned to two inequivalent ^{55}Mn nuclei both having spin $I = 5/2$ (12). A theoretical stick spectrum and a computer-generated spectrum for a Mn(III)Mn(IV) pair with $S = 1/2$ are given in Fig. 4D and C, respectively. Hyperfine coupling through second order to two inequivalent $I = 5/2$ nuclei is included. The hyperfine constants used are $|A_1| = 177$ G and $|A_2| = 82$ G, and the linewidth was assumed to be gaussian with a linewidth Δ increasing with the nuclear spin quantum number $\Delta = \Delta_0 + b_1 m_1 + b_2 m_2$; $\Delta_0 = 15$ G, $b_1 = 1.7$ G, and $b_2 = 0.8$ G. These values were chosen to optimize agreement with Fig. 4B. Further details of the simulation are given in ref. 12. The agreement with the prominent 16 hyperfine lines is noted. It is expected that further improvement in the lineshapes could be realized by including hyperfine and g -tensor anisotropies. The inequivalence of the hyperfine couplings shows that the valence is trapped so that distinguishable oxidation states for the Mn ions are present.

An antiferromagnetically coupled pair is expected to have hyperfine constants determined by the projection of the individual ion spins on the total spin, $\hat{S}_1 \cdot \hat{S} / S^2$. For a Mn(III)Mn(IV) pair this gives $A_1 = 2A_1'$ and $A_2 = -A_2'$, in which A_1' and A_2' are the values for isolated Mn(III) and Mn(IV) ions, respectively (12). The literature value (13) for Mn(IV) in Al_2O_3 is $A_2' = 75$ G, and the Mn(III) value is expected to be intermediate between Mn(II) and Mn(IV) , $A_1' \approx 80\text{--}90$ G. These values are in good agreement with the coupling constants used in the simulation in Fig. 4C.

The EPR signal produced on the first flash should be identified predominantly with the S_2 metastable state in Kok's model for the photocycle of the water-oxidation enzyme (2). Indirect EPR analysis of the amount of $\text{Mn(H}_2\text{O)}_6^{3+}$ released from chloroplasts following light flashes and heat shock has been interpreted as evidence for identifying S_2 with a Mn(III)Mn(III) dimer (9). Such a dimer will be EPR silent because of the paired spins and cannot account for our results.

The chloroplast spectrum in Fig. 4B exhibits peaks outside of the region of the prominent 16 hyperfine lines found for the synthetic dimer. This is shown by the vertical lines. The one additional peak on the low-field side is unexpectedly intense com-

pared to the neighboring hyperfine peaks in the multiline spectrum, which suggests that it may be due, in part, to a different paramagnetic center. Two weak peaks are found on the high-field side in a region where interference from other paramagnetic centers is not expected. The weak intensity and poor resolution make assignment of these peaks difficult. Nevertheless, they suggest that the hyperfine structure may extend well beyond that expected for a simple (III,IV) dimer.

Two explanations can account for the additional peaks in the wings of the spectrum. A Mn(II,III) dimer should have a wider overall spectrum because the hyperfine constants are larger— $A_1 = 7/3 A_1'$ for Mn(II); $A_2 = -4/3 A_2'$ for Mn(III). A stick spectrum for this dimer, using hyperfine values in the range of these predictions, is given in Fig. 4E. Although the positions of the outside lines are correctly predicted, there is little agreement with the interior lines (compare Fig. 4D and E). These results indicate that a (II,III) dimer cannot explain the EPR spectrum in chloroplasts. The synthetic dimers are unstable in the (II,III) oxidation state, evidently because Mn(II) possesses no ligand field stabilization energy.

The peaks in the wings of the spectrum might be caused by a mixed-valence cluster of four Mn ions having three Mn(III) and one Mn(IV) which are coupled by spin exchange. The number of possible spin states available to such a tetramer is $\pi_{i=1}^4 (2S_i + 1) = 500$. It is necessary to establish which states would be occupied at the temperatures of our measurements (7.5–35 K) and what hyperfine structure each state should possess. The EPR results indicate that the manifold with total spin $S = 1/2$ must be lowest because the higher spin states would possess zero-field splittings and hence would not have the nearly isotropic features observed in Fig. 4. When this manifold lies lowest, net antiferromagnetic coupling exists, although individual pairs may be ferromagnetically coupled. The electronic spin of each Mn ion must have a projection on the total spin of the cluster in order for each ion to contribute to the observed hyperfine structure. For one of the nuclei, the isotropic hyperfine interaction is given by

$$A_i \tilde{S} \cdot \tilde{I}_i = \left(\frac{A_i \tilde{S}_i \cdot \tilde{S}}{\tilde{S}^2} \right) \tilde{S} \cdot \tilde{I}_i \quad [1]$$

in which I_i refers to the nuclear spin of the i^{th} nucleus. The projection $\tilde{S}_i \cdot \tilde{S}/\tilde{S}^2$ could be evaluated by calculation of the expectation value of this operator for every state that is occupied. This requires a knowledge of the spin functions of the cluster, which are tedious to evaluate. However, a simpler approach is feasible for clusters possessing some symmetry which permits $\tilde{S}$ to be evaluated by vector coupling rules. This approach will now be outlined.

In the general case there can be a maximum of six different pairwise exchange interactions between four paramagnetic ions with total exchange energy given by the solution to the corresponding Heisenberg hamiltonian:

$$-2 \sum_{j>i=1}^4 J_{ij} S_i S_j \quad [2]$$

Only pairwise interactions and quadratic exchange needs to be considered in this simple model. When the exchange interactions are very much larger than the hyperfine, Zeeman, and zero-field interactions of the individual ions, the total spin of the cluster, $\tilde{S} = \sum \tilde{S}_i$, is quantized along the external magnetic field. The hyperfine couplings expected from such a cluster can then be calculated in a straightforward manner by extension of the simpler results for a dimer (12). It first is necessary to show that the ground state has $S = 1/2$. If the cluster had cubic symmetry, then this manifold would be 16-fold degenerate. In clusters of

lower symmetry this degeneracy is reduced.

We shall take a specific example to illustrate the expected hyperfine constants. The case of a (III,III) dimer exchange coupled to a (III,IV) dimer has a maximum possible symmetry of C_{2v} and may be symbolically represented by

Here, $J_{12} = J_{23} = J_{34} = J_{14} = J$; $J_{13} \neq J_{24}$. Eq. 2 can be solved for the energy for this special case to yield (14):

$$E(S, S_{13}, S_{24}) = -JS(S+1) + (J - J_{13})S_{13}(S_{13}+1) + (J - J_{24})S_{24}(S_{24}+1) + J_{13}[S_1(S_1+1) + S_3(S_3+1)] + J_{24}[S_2(S_2+1) + S_4(S_4+1)] \quad [3]$$

in which

$$S_{13} = S_1 + S_3, S_1 + S_3 - 1, \dots, |S_1 - S_3| = 4, 3, \dots, 0$$

$$S_{24} = S_2 + S_4, S_2 + S_4 - 1, \dots, |S_2 - S_4| = 7/2, 5/2, \dots, 1/2$$

$$S = S_{13} + S_{24}, S_{13} + S_{24} - 1, \dots, |S_{13} - S_{24}|$$

$$= 15/2, 13/2, \dots, 1/2.$$

This equation shows that only S , S_{13} , and S_{24} have quantized values. It also shows that the $S = 1/2$ manifold is lowest provided that J is negative; that is, there exists antiferromagnetic coupling between four of the six possible pairs. The 16 states within the $S = 1/2$ manifold split into degenerate pairs (Kramer's doublets) with their order determined by the signs of the terms $(J - J_{13})$ and $(J - J_{24})$. If both terms are negative, the maximum values of the coupled spins S_{13} and S_{24} characterize the lowest state. All $S = 1/2$ doublets which are populated should be observable by EPR; however, we will make the simplifying assumption that only the lowest doublet is populated at 7.5 K.

The projections of the individual spins $\tilde{S}_i$ on $\tilde{S}$ are determined by the projection of $\tilde{S}_i$ on its quantized resultant $\tilde{S}_{ij}$ and, in turn, by the projection of $\tilde{S}_{ij}$ on the total spin

$$A_i = \frac{\tilde{S}_i \cdot \tilde{S}_{ij}}{\tilde{S}_{ij}^2} \times \frac{\tilde{S}_{ij} \cdot \tilde{S}}{\tilde{S}^2} \times A_i' \quad [4]$$

A similar expression is valid for A_j . The vector coupling rules give, for example,

$$A_1 = \frac{A_1'}{4} \times \left(\frac{S_{13}(S_{13}+1) + S_1(S_1+1) - S_3(S_3+1)}{S_{13}(S_{13}+1)} \right) \times \left(\frac{S(S+1) + S_{13}(S_{13}+1) - S_{24}(S_{24}+1)}{S(S+1)} \right) \quad [5]$$

Inspection of Eq. 4 shows that, if the ground state has $S_{13} = 0$ and $S_{24} = 1/2$, then only two of the four Mn ions exhibit hyperfine coupling and the values are identical to the (III,IV) dimer. However, if the ground state has the maximum values $S_{13} = 4$ and $S_{24} = 7/2$, the hyperfine constants are $5/3 A_1'$, $5/3 A_2'$, and $-4/3 A_3'$ for Mn(III) and $-A_4'$ for Mn(IV).

The predicted overall breadth of the hyperfine structure is $5(|A_1| + |A_2| + |A_3| + |A_4|) \approx 2,358 \pm 75$ G by using the literature values for the monomer ions and these results. The experimentally observed width of the 16 prominent lines is 1328 ± 35 G and the additional lines in the wings increase this to at least 1647 G. There may be a wider spectral breadth which is not apparent due to the finite signal/noise ratio that we could achieve.

This simple model for the hyperfine constants indicates that

the large overall width of the chloroplast spectrum might be caused by a cluster of four Mn ions. A detailed computer simulation and higher sensitivity measurements will be needed in order to test this preliminary interpretation more firmly.

In clusters of symmetry, lower than C_{2v} , which are perhaps more realistic for biological samples, any of the eight doublets in the $S = 1/2$ manifold may be lowest in energy, and each has different hyperfine constants. Numerical methods can be used to solve for the state energies in these more complicated cases; however, the possible hyperfine constants will be limited by the above extreme cases and no new features arise. The temperature dependence of the spectrum under increased sensitivity may test this interpretation by revealing the population of different Kramer's doublets within the $S = 1/2$ manifold.

The intensity changes given in Fig. 2 are consistent with a model in which the cluster is in an initial (dark) oxidation state with all ions in equivalent oxidation states and spin paired. Because $S = 0$ this will be EPR silent. The first flash extracts one electron from the metal ions to yield an $S = 1/2$ cluster which is EPR observable. The second flash decreases the intensity appreciably which might be caused by removal of one electron from another metal ion to yield an $S = 0$ cluster again, or by oxidation of a directly coordinated ligand, such as water, or oxidation of a neighboring cofactor which is sufficiently close to the Mn cluster to produce line broadening by cross relaxation. The intensity changes on flashes 3 and 4 are small, indicating that oxidation state change of the Mn ions are not EPR detectable on these steps. If oxidation of the Mn ions of a tetramer were involved on flash 3, an EPR active state with $S = 1/2$ might be expected from a cluster with three Mn(IV) and one Mn(III) ions. Because this is not found, oxidation of ligands or other cofactors seems to be occurring on flash 3.

The EPR intensity for the multiline signal does not decrease to zero on flashes 2, 3, and 4, and it reaches a steady state after many flashes. This is probably due to the existence of more than a single S_n state in the dark and to double hits and misses with each flash as has been suggested for the analogous behavior of the O_2 yield per flash (2). Flash-dependent changes in the EPR intensity of chloroplast Mn(II) observed at room temperature (7) are not the same as the signal reported here. It is possible that

these flash-induced Mn(II) EPR signal changes at room temperature may involve secondary effects associated with electron transport unrelated to water oxidation.

CONCLUSIONS

These results strongly suggest the involvement of a cluster containing at least two, and possibly four, Mn ions in photosynthetic water oxidation. Mn(III) and Mn(IV) oxidation states are consistent with the hyperfine data but Mn(II) is not. The variation in the EPR intensity with flash number indicates that both the first and second flashes oxidize the Mn cluster and that the initial oxidation state of the Mn cluster is regenerated on the fourth flash.

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