Parallel Polarization EPR Detection of an S_1-State "Multiline" EPR Signal in Photosystem II Particles from *Synechocystis* sp. PCC 6803

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Photosystem II (PS II) oxygen evolution occurs through a cycle of five "S-state" intermediates, S_0 through S_4, where the subscript represents the number of oxidizing equivalents abstracted from the PS II *oxygen evolving complex* (OEC) by the photooxidized $P680^+$ Chl moiety.[1,2] The OEC consists of a tetranuclear Mn cluster, the redox-active tyrosine Y_Z, and the essential cofactors Cl^- and Ca^{2+}.[2] Extensive EPR characterization of the odd-electron S_2 state has led to much of our current knowledge of the structure, protein ligation, and substrate and inhibitor binding modes of the Mn cluster.[2,3] However, EPR characterization of the more reduced S_0- and S_1-states of the OEC has remained more elusive.

Given the odd-spin magnetic properties of the S_2-state, the S_1-state must have an even number of electrons partitioned among the four Mn ions of the OEC. If these electrons are distributed among the ions of a single exchange-coupled tetranuclear cluster, such as observed for the odd-electron S_2-state,[3d−g] one would expect an energy ladder of integer spins states, including the diamagnetic $S = 0$ state. The effective spin values of the ground and excited states of the coupled cluster will cover the range between 0 and a maximum value, S_{max}, which depends on the individual Mn oxidation states present in the S_1-state configuration.[4] Depending on the temperature and the magnitudes of the energy gaps within the ladder of the coupled cluster, some of these excited states may join the ground state in contributing to the magnetic properties of the cluster. Alternatively, the S_1-state could consist of lower order magnetic units, such as a pair of noninteracting dinuclear clusters, a trinuclear cluster with a noninteracting monomer, a dinuclear cluster with a pair of noninteracting monomers, or even four noninteracting monomers. Thus, characterization of the magnetic properties of the S_1-state is important for our understanding of the structure of the OEC.

For integer spin systems with nonnegligible zero-field splitting interactions, the EPR experiment, performed with the oscillating magnetic field $\vec{B}_1$ polarized parallel to the static magnetic field $\vec{B}_0$, can provide for sensitive detection of "$\Delta M_S = 0$" electron spin transitions.[5] Dexheimer and Klein[6] applied such parallel polarization EPR spectroscopy to the study of the OEC in PS II-enriched spinach thylakoid membranes and reported a broad (600 G peak-to-peak width), featureless EPR signal centered about the $g = 4.8$ region of the spectrum, which they assigned to an $S = 1$ spin state present in the S_1-state. This spectrum bears appreciable similarity to the parallel polarization EPR spectrum reported from a synthetic 2Mn(III)2Mn(IV) cluster.[7] However, no ^{55}Mn hyperfine structure is observed on the S_1-state EPR signal, despite the resolution of ^{55}Mn hyperfine features in the parallel polarization EPR spectra of some integer spin Mn(III) complexes.[8] Thus, although the disappearance of the signal upon illumination is correlated with the appearance of the S_2-state multiline EPR signal, the most direct indication of a manganese origin of the signal, the detection of ^{55}Mn hyperfine features, is absent. Confirmation of this integer spin EPR signal in other laboratories has been slow in coming,[2c] but recently Yamauchi et al.[9] provided a detailed account of their reproduction of this signal and its more complete characterization.

In this paper, we present the results of parallel polarization EPR studies performed on PS II particles isolated[10] from the cyanobacterium *Synechocystis* sp. PCC 6803. The trace labeled "dark" in Figure 1a displays the parallel polarization EPR spectrum of the *Synechocystis* PS II preparation in the S_1-state. A "multiline" EPR spectrum is clearly evident. Following advancement to the S_2 state by sample illumination at 195 K, confirmed by the generation of the conventional perpendicular polarization $g = 2$ multiline EPR signal[3a] (Figure 1b, "illuminated minus dark"), the amplitude of this parallel polarization multiline signal is greatly reduced (Figure 1a, trace labeled "illuminated"). The "dark minus illuminated" parallel polarization difference spectrum consists of a well-resolved multiline EPR spectrum centered at an effective g-value of approximately 12 with at least 18 well-resolved hyperfine lines with an average splitting of 32 G.[11] In exactly equivalent fashion to the conclusions generated by the observation of such multiline hyperfine structure in the $g = 2$ and $g = 4.1$ S_2-state signals,[3a,d,e] this hyperfine pattern provides unambiguous evidence for the existence of a multinuclear exchange-coupled paramagnetic Mn cluster in the S_1-state of *Synechocystis* PS II particles.

Dexheimer and Klein[6] and Yamauchi et al.[9] noted that the spectrum assigned to the S_1-state in spinach PS II preparations could arise from the first excited state of a dinuclear Mn(III)-Mn(III) cluster. However, this would not appear to be a sufficient "minimal unit" to explain the highly structured EPR signal that

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(11) The features in the 700−1200 G range are present in Mn-depleted PS II particles and therefore cannot be assigned to the S_1 state of the Mn cluster. Precise zero-field splittings will be required to relate the observed signal intensity to the fraction of PS II centers giving rise to the signal, but the fact that the signal amplitude is comparable to the conventional S_2 multiline signal provides qualitative evidence that it arises from a majority of centers.

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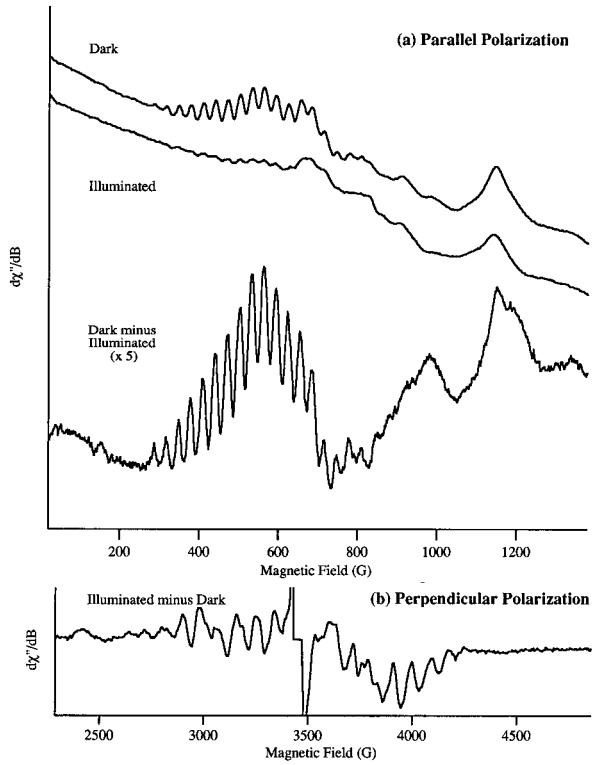

Figure 1. (a) Trace "dark": the parallel polarization EPR spectrum of *Synechocystis* 6803 PS II particles dark adapted for 1.5 h following 195 K preillumination. Trace "illuminated": the spectrum following 195 K illumination (6 m) to drive the $S_1 \rightarrow S_2$ transition. Trace "dark minus illuminated": The dark minus illuminated difference spectrum ($\times 5$). No baseline manipulations[6,9] were employed. (b) Trace "illuminated minus dark": the illuminated minus dark perpendicular polarization spectrum of the same states. The $g = 2$ multiline signal is partially saturated under these conditions. Experimental conditions: Spectrometer, Bruker ECS106, (a) parallel TE 012 mode and (b) perpendicular TE 102 mode of Bruker ER 4116DM cavity; microwave frequency, (a) 9.41 and (b) 9.67 GHz; microwave power, (a) 80.3 and (b) 3.2 mW; temperature, 3.6 K; modulation amplitude, (a) 8 and (b) 10 G; modulation frequency, 100 kHz. PS II preparation: *Synechocystis* 6803 PS II particles were isolated essentially as described by Tang and Diner[10] and contained 40 ± 2 Chl *a* per photoreducible. Sample composition: 5−6 mg Chl/mL in 50 mM MES−NaOH (pH = 6.0), 20 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM $MgSO_4$, 0.03% *n*-dodecyl-β-D-maltoside (Anatrace, Maumee, OH), 25% (v/v) glycerol (Gibco-BRL). Sample activity: 5100 ± 200 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

we observe in *Synechocystis* PS II particles. The ^{55}Mn hyperfine splitting observed for Mn(III) mononuclear and dinuclear clusters is greater than 50 G,[8] appreciably greater than the 32 G splitting observed here. More importantly, the hyperfine interactions for the two Mn ions of either Mn(III)Mn(III) or Mn(IV)Mn(IV) dinuclear clusters would be expected to be approximately equivalent, giving rise to 11-line EPR spectra,[12] such as experimentally observed for Mn(III)Mn(III) clusters.[8] In contrast, our S_1-state signal consists of at least 18 hyperfine lines, suggesting that at least three of the four Mn ions of the OEC must be exchange-coupled in the S_1-state to give rise to this integer spin EPR signal.[13] We note that antiferromagnetic exchange-coupled Mn(II)Mn(II) dinuclear clusters can give rise to very complex EPR line shapes with more than 11 distinct transitions.[14] However, these complex line shapes arise from a superposition of excited paramagnetic spin states energetically positioned above the diamagnetic ground state. This leads to dramatic temperature dependencies of the spectra, including a disappearance of the spectra at the lowest temperatures where only the diamagnetic ground state is significantly populated. It is clear that the Mn cluster is not sufficiently reduced in the S_1-state to contain a pair of Mn(II) ions.[2] However, antiferromagnetically coupled Mn(IV)Mn(IV) or Mn(III)Mn(III) pairs would also exhibit excited paramagnetic integer spin states, but given the short 2.7 Å Mn−Mn separation determined from EXAFS of the S_1-state,[2] these states would be at higher energies[15] than those for the Mn(II)-Mn(II) clusters. We have examined the temperature dependence of the S_1-state multiline signal over the temperature range of 3.6−9.0 K, and the signal amplitude increases with decreased temperature in a Curie law ($1/T$) fashion, demonstrating that the signal arises from an integer spin ground state or a very low-lying excited state.[16] Moreover, the signal line shape is unchanged over this temperature range, showing no evidence of contributions from multiple spin manifolds. These results are not consistent with possible Mn(III)Mn(III) or Mn(IV)Mn(IV) origins for the S_1-state signal, and we conclude that at least three of the four Mn ions of the OEC must be exchange-coupled in the S_1-state to give rise to this integer spin EPR signal. We favor a tetranuclear cluster assignment, because if only three Mn ions were coupled to give rise to this integer spin S_1 multiline signal, the remaining Mn ion would also have to be integer spin, therefore Mn(III), which should be detectable as an additional 6-line parallel polarization EPR signal.[8,17]

In conclusion, the observation of this S_1-state "multiline" signal provides conclusive evidence for a paramagnetic tri- or tetranuclear Mn cluster in the S_1-state of the OEC, with the tetranuclear assignment being most consistent with the overall data. Moreover, this highly structured EPR signal, with at least 18 resolved ^{55}Mn hyperfine lines, will provide a new spectroscopic handle to study the S_1-state. There are also recent reports of an S_0-state multiline signal, generated either through chemical reduction[18a] or via three-flash forward turnover.[18b,c] Therefore, much progress is now being made in the characterization of the more reduced states of the PS II Mn cluster.

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(12) The number of hyperfine lines for a system with k different classes of n_i equivalent spin I_i nuclei is $\Pi^k_{i=1}(2n_iI_i+1)$.

(13) We can also rule out a trinuclear cluster with three equivalent hyperfine couplings, because such a cluster would give rise to a 16-line spectrum. However, even if all three Mn ions of a trinuclear cluster were trapped in the same oxidation state, such as 3Mn(III), inequivalent exchange coupling pathways between the three ions could lead to inequivalences in the effective hyperfine tensors of the coupled cluster.

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