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The S_0 State of the Oxygen-Evolving Complex in Photosystem II Is Paramagnetic: Detection of an EPR Multiline Signal

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The photosynthetic oxidation of water to molecular oxygen is energetically driven by light-induced charge separations in the reaction center of photosystem II (PS II). The reaction is catalyzed by a tetranuclear manganese cluster contained in the oxygen-evolving complex (OEC). The OEC cycles through five different redox states termed S_0 to S_4, with S_1 being the dark-stable state. Oxygen is released during the $S_4 \rightarrow S_0$ transition.[1] The removal of one electron from the OEC on each S state transition leads to the idea that alternate S states should be paramagnetic because of their odd-electron number. The multiline EPR signal, which is the hallmark of the S_2 state, establishes the odd-electron character of the Mn cluster in S_2.[2] The S_1 state, one-electron reduced from S_2, is paramagnetic but of even electron number, and a non-Kramers EPR signal is observed in parallel-polarized EPR.[3] Because the S_0 state is reduced by one further electron, it is expected to be an odd-electron or Kramers state observable with conventional EPR. Hence, it was somewhat surprising that no EPR signal had been reported for this state. This problem was recently resolved by Messinger et al. who observed a new EPR multiline signal in an S_0* state, an S_0-like state produced by reduction of the S_1 state by hydroxylamine or hydrazine.[4] The essential ingredient was the addition of 1.5% methanol. We now report the observation of this EPR signal in a physiological S_0 state produced by three-flash illumination of dark-adapted PS II membranes. This EPR signal is sufficiently similar to that produced by NH_2OH treatment so that, from the perspective of EPR, one need no longer distinguish the states prepared by the two methods. Furthermore, we describe a broad EPR signal for the S_0 state in absence of methanol.

Dark-adapted spinach PS II membranes[5] were enriched in S_0 by the following flash procedure: aliquots were illuminated at a chlorophyll (Chl) concentration of 1 mg/mL in ice-cold pH 6.5 buffer (5 mM $CaCl_2$, 5 mM $MgCl_2$, 15 mM NaCl, 50 mM MES, 400 mM sucrose) with one preflash (Xe flash lamp, 13 μs FWHM, 5 J per pulse; pathlength ∼ 2 mm), further dark-adapted on ice for 90−120 min, and illuminated with three Xe flashes (0.5 Hz). Before centrifugation (30 min, 40 000 × *g*, 4 °C) 1.5% methanol (v/v), 20 μM phenyl-*p*-benzoquinone (PPBQ; 50 mM in methanol or DMSO),[6] and/or 2.5 μM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP; 2.5 mM in ethanol) were added as indicated in Figure 1. The pellets (∼30 mg Chl/mL) were transferred in the dark into special EPR Lucite holders of 120 μL volume and frozen in liquid N_2. FCCP was used to accelerate the deactivation of the S_2 and S_3 states of PS II to S_1[7] and to reduce the stable tyrosine radical of PS II,[8] Y_D^{ox}, which prevents the reaction $S_0 + Y_D^{ox} \rightarrow S_1 + Y_D^{red}$.

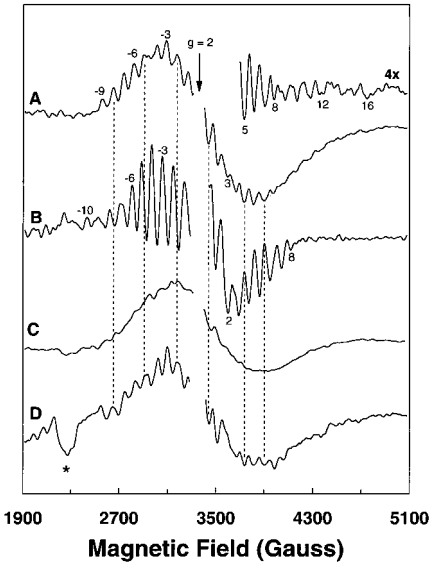

Figure 1. X-band EPR difference spectra from PS II membranes at 7 K: A, physiological S_0 state minus S_1 state in presence of methanol (1.5%, v/v), FCCP and PPBQ; the inset (4× amplified) was obtained after subtraction of the broad underlying signal; B, S_2 state minus S_1 with same additions, induced by 200 K continuous illumination (6 min); C, S_0 state minus S_1, both without methanol, but with PPBQ and FCCP addition; D, S_0* state induced by NH_2OH incubation in presence of 1.5% methanol (v/v) and 1 mM EDTA minus S_1 with the same additions. For clarity, spectrum D is amplified by a factor of 3 relative to the other spectra; the asterisk at the spectrum labels a subtraction artifact that is probably due to different amounts of low-potential cytochrome b_{559}^+ in the S_0* sample compared to the S_1 control. The $g = 2$ regions containing the Y_D^{ox} radical signal were deleted for clarity. All measurements were recorded using a Varian E-109 X-band spectrometer with an E-102 microwave bridge and an Air Products helium flow cryostat. Instrument conditions: microwave power, 30 mW; modulation frequency, 100 kHz; modulation amplitude, 20 G; time constant, 0.5 s; scan time, 4 min.

Figure 1A shows an EPR difference spectrum from the S_0 sample (minus S_1) prepared with FCCP, PPBQ, and methanol. A multiline signal clearly different from the well-known S_2 multiline signal (Figure 1B, same additions) is observed. Most of the peaks are out of phase between the two signals (see dashed lines in Figure 1). The average splitting of the hyperfine lines is very similar, about 85−90 G, but the values for the S_0 signal are more variable (70−110 G) than those for the S_2 multiline (80−100 G). Although it is difficult at the current signal-to-noise ratio to identify clearly the last peak at the high-field side of the S_0 multiline signal, a careful study of the outer wings (see inset in Figure 1A) and a comparison of spectra obtained from several independent samples (data not shown) show that the total spectral breadth is 2200−2400 G and the total number of peaks is 24−26, compared to 18−20 reported for S_2.[2,9] Therefore, the S_0 multiline is about 300−500 G wider

† Structural Biology Division.
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than the S_2 multiline signal. This extra width is exclusively on the high-field side of the S_0 multiline which gives rise to an asymmetry of this EPR signal, indicative of an average g value below $g = 2.0$. In the absence of FCCP, mixtures of S_0 and S_2 multilines were observed, which displayed in their outer low-field wings peaks of the S_0 multiline (data not shown). This indicates that the S_0 multiline can be generated in the absence of FCCP. The S_0 state concentration of sample A (Figure 1) is about 50%. This was determined by converting the residual S_1 state population into S_2 by 200 K illumination and comparing the resulting S_2 multiline amplitude with that of sample B (Figure 1).[10] The S_0 minus S_1 difference spectrum obtained in the absence of methanol is shown in Figure 1C. A broad ~2400 G wide signal with only poorly resolved hyperfine structure is observed, showing (i) that methanol is important for observing the hyperfine lines and (ii) that S_0 has an EPR signal also in the absence of methanol. The effect of methanol on the amplitude of the hyperfine peaks may be explained by the hypothesis that it can bind at or near the Mn cluster and change the hyperfine couplings of the involved Mn ions. Support for this speculation comes from the recent finding that methanol binds to a binuclear Mn(III,IV) complex.[11] Alternatively, methanol may simply reduce the hyperfine line width through a reduction of inhomogeneity around the Mn cluster. Figure 1D displays a S_0* multiline signal, from a sample prepared using NH_2OH as reductant.[4] Only minor differences (if any) can be seen between the S_0 and S_0* state multiline signals. On the basis of this finding, we propose that the two states are identical.

The S_2 state has under certain conditions a second EPR signal at $g = 4.1$.[9,12] We therefore took difference spectra in the field range of 400−2400 G for the different S_0 samples (±methanol). No indications for a $g = 4.1$ signal were found in any of the samples, but all displayed small reproducible changes at higher g values. These require further study to clarify their origin.

On the basis of comparison with X-ray absorption edge positions and shapes for model complexes, the following Mn redox states have been proposed for the S_0 state: (II,III,IV_2) and (III_3,IV).[1,9] It has been observed for binuclear Mn complexes[13] and in Mn catalase[14] that the spectral width of the EPR multiline signals is greater for the (II,III) than for the (III,-IV) forms. The larger spectral width of the S_0 multiline compared to the S_2 multiline might therefore be indicative of a Mn^{II} center in the S_0 state.[4] Simulations of the S_0 EPR multiline signal using previously employed values of the projected Mn hyperfine constants (Mn^{II}, $A' = 85-100$ G; Mn^{III}, $A' = 80-95$ G; Mn^{IV}, $A' = 70-85$ G)[14,15] were performed using second-order perturbation theory.[2,16] The results for both binuclear and a C_{2v} symmetric tetranuclear species (see Scheme 1) with various combinations of oxidation states are presented in Table 1. Assuming a tetranuclear origin for the S_0 multiline, it was possible to simulate the main features of the S_0 multiline, i.e., the number of lines, the spectral width, and the relatively weak hyperfine structure on top of a broad signal, within this simple model by using the average of any set of the calculated parameters (Table 1). The current level of simulation does not allow us a distinction between the (II,III_3), (II,III,IV_2), and (III,IV_3) oxidation states. In contrast, we have not been able to achieve satisfactory simulations, especially of the broad "underlying" feature, assuming isotropic (see Table 1) or axially symmetric g and A values for the (II,III) or (III,IV) binuclear clusters. However, in rhombic simulations with large anisotropic g and A values, these features could be largely reproduced (see the Supporting Information for examples of EPR simulations). Measurements at other microwave frequencies can reveal the degree of anisotropy in the S_0 EPR multiline signal and allow a distinction between the different models.[17]

Scheme 1. Spin Coupling Scheme Used To Obtain the Spins S_{13} and S_{24} in the Vector Coupling Approach

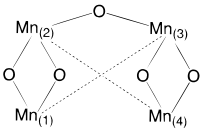

Table 1. Theoretically Predicted Isotropic Hyperfine Constants and Spectral Widths for Different Mixed-Valence Mn Binuclear and Tetranuclear Clusters[a]

Binuclear Cluster

parameter	(II,III)	(III,IV)
$\|A_1\|$ (G)	$7/3A_1' = 198-233$	$2A_1' = 160-190$
$\|A_2\|$ (G)	$4/3A_2' = 107-127$	$A_2' = 70-85$
width (G)	1525−1800	1150−1375

Tetranuclear Cluster

parameter	(II,III,III,III)	(II,III,IV,IV)	(III,III,III,IV)
$\|A_1\|$ (G)	$55/27A_1' = 173-204$	$25/12A_1' = 177-208$	$5/3A_1' = 133-158$
$\|A_2\|$ (G)	$4/3A_2' = 107-127$	$4/3A_2' = 107-127$	$5/3A_2' = 133-158$
$\|A_3\|$ (G)	$44/27A_3' = 130-155$	$5/4A_3' = 87-106$	$4/3A_3' = 107-127$
$\|A_4\|$ (G)	$4/3A_4' = 107-127$	$A_4' = 70-85$	$A_4' = 70-85$
width (G)	2585−3065	2205−2630	2215−2640

[a] For the tetranuclear cluster, the numbers in parentheses give the Mn oxidation states in the order $Mn_{(1)}$, $Mn_{(2)}$, $Mn_{(3)}$, and $Mn_{(4)}$ as depicted in Scheme 1. The $|A|$ values were derived from the A' values given in the text and from a general formula for the isotropic hyperfine coupling constants $A_i = A_i'(\mathbf{S}_i\cdot\mathbf{S}_{ij}/\mathbf{S}_{ij}^2)(\mathbf{S}_{ij}\cdot\mathbf{S}/\mathbf{S}^2)$ for the Mn clusters,[2,16] with $i,j = 1-4$ for a tetranuclear species (see Scheme 1 for an illustration of the spin coupling scheme). The spectral width was calculated according to $5(|A_1| + |A_2|)$ for the binuclear systems and $5(|A_1| + |A_2| + |A_3| + |A_4|)$ for tetranuclear clusters.

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Supporting Information Available: Simulated EPR spectra (9 pages). See any current masthead page for ordering and Internet access instructions.

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