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The S₀ 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 S₀ to S₅, with S₀ being the dark-stable state. Oxygen is released during the S₁ → S₀ transition. 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 S₂ state, establishes the odd-electron character of the Mn cluster in S₂. The S₀ state, one-electron reduced from S₂, is paramagnetic but of even electron number, and a non-Kramers EPR signal is observed in parallel-polarized EPR. Because the S₀ 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 S₀ state, an S₀-like state produced by reduction of the S₁ state by hydroxylamine or hydrazine. The essential ingredient was the addition of 1.5% methanol. We now report the observation of this EPR signal in a physiological S₀ state produced by three-flash illumination of dark-adapted PS II membranes. This EPR signal is sufficiently similar to that produced by NH₂OH 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 S₀ state in absence of methanol.

Dark-adapted spinach PS II membranes were enriched in S₀ 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 CaCl₂, 5 mM MgCl₂, 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), the pellets were resuspended in 2 mM phenyl-p-benzoquinone (PPBQ; 50 mM in methanol or DMSO), 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 membrane holders of 120 μL volume and frozen in liquid N₂. FCCP was used to accelerate the deactivation of the S₁ and S₅ states of PS II to S₀ and to reduce the stable tyrosine radical of PS II, Y₁(red), which prevents the reaction S₀ + Y₁(red) → S₁ + Y₁(red).

Figure 1A shows an EPR difference spectrum from the S₀ sample (minus S₁) prepared with FCCP, PPBQ, and methanol. A multiline signal clearly different from the well-known S₂ 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 S₀ signal are more variable (70 – 110 G) than those for the S₂ 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 S₀ 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 S₂. Therefore, the S₀ multiline is about 300 – 500 G wider.
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 $S_2$ multlines 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 $S_0$ 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 $\sim 2400$ 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(II,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$_4$OH 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,IV)$_2$.$^{13}$ It has been observed for binuclear Mn complexes$^3$ and in Mn catalase$^8$ that the spectral width of the EPR multiline signals is greater for the (II,III) than for the (II,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.$^3$ Simulations of the $S_0$ EPR multiline signal using previously employed values of the dipolar 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$_4$ symmetric tetranuclear species (Scheme 1) with various combinations of oxidation states are presented in Table 1. Assuming a tetranuclear origin for the $S_0$ multiline, it was

### Scheme 1. Spin Coupling Scheme Used To Obtain the Spins $S_0$ and $S_2$ in the Vector Coupling Approach

![Diagram](image)

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(II,III,III)</th>
<th>(II,III,IV)</th>
<th>(III,III,IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$</td>
<td>A_1</td>
<td>(G)$</td>
<td>7/34</td>
</tr>
<tr>
<td>$</td>
<td>A_2</td>
<td>(G)$</td>
<td>4/34</td>
</tr>
<tr>
<td>Width (G)</td>
<td>1525–1800</td>
<td>1150–1375</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ For the tetranuclear cluster, the numbers in parentheses give the Mn oxidation states in the order Mn$_{II_1}$, Mn$_{III_2}$, Mn$_{III_3}$, and Mn$_{IV_4}$, 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 $A = A' S_{i} S_{j} S_{k} S_{l}$ for the Mn clusters, $^{2,16}$ with $ij = 1–4$ for a tetranuclear species (see Scheme 1 for an illustration of the spin coupling scheme). The spectral width was calculated according to $S(|A_1| + |A_2|)$ for the binuclear systems and $S(|A_1| + |A_2|)$ for the tetranuclear clusters.

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,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.$^{17}$

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