

## <sup>55</sup>Mn Pulse ENDOR at 34 GHz of the S<sub>0</sub> and S<sub>2</sub> States of the Oxygen-Evolving Complex in Photosystem II

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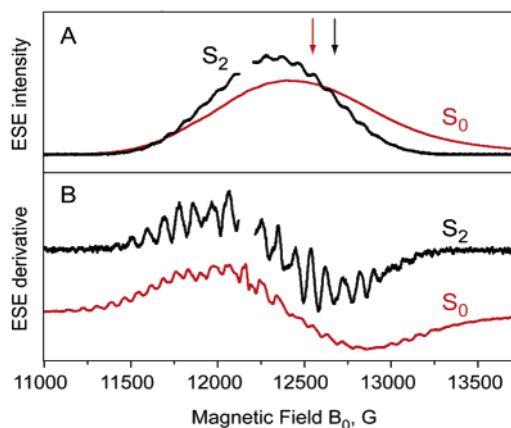
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Powered by light-induced charge separations that generate oxidizing equivalents of about +1.3 V, photosystem II (PSII) has the unique capability to oxidize water to molecular oxygen. Water splitting is catalyzed by a Mn<sub>4</sub>O<sub>x</sub>Ca complex housed in a special protein environment that also controls proton movements and the access of water. This functional unit of PSII is referred to as the oxygen-evolving complex (OEC). PSII created the aerobic atmosphere on earth and may serve as a model for technical approaches to split water by sunlight, which is a prerequisite for a sustainable hydrogen economy. Despite the availability of crystal structures of PSII with 3.5 and 3.2 Å resolutions and of extensive EPR and EXAFS studies, the precise geometric and electronic structures of the various functional states of the Mn<sub>4</sub>O<sub>x</sub>Ca complex are still unknown.<sup>1–5</sup> During the reaction sequence (Kok cycle) the OEC passes through five oxidation states (S states, S<sub>0</sub>–S<sub>4</sub>),<sup>6</sup> of which the S<sub>0</sub> and S<sub>2</sub> states have *S* = 1/2 ground states. Continuous wave (CW) EPR has been used to study the electronic structures of these states,<sup>7–10</sup> but despite the rich hyperfine structures of the S<sub>2</sub> and S<sub>0</sub> EPR multiline signals, it has not been possible to uniquely determine the hyperfine interaction (HFI) tensors of the individual Mn ions in this way. For such complex spin systems, it is required to directly probe the Mn HFI parameters by <sup>55</sup>Mn pulse ENDOR spectroscopy. For technical reasons and due to the complex preparation procedure for concentrated S<sub>0</sub> state samples, such experiments were done so far only at X-band for the S<sub>2</sub> state; they gave the first reliable HFI parameters.<sup>11</sup> In this study we report <sup>55</sup>Mn pulse ENDOR spectra for the S<sub>0</sub> and S<sub>2</sub> states at Q-band frequency (34 GHz). Numerical analysis of our spectra (i) shows that all four Mn ions are magnetically coupled, (ii) allows a refinement of the HFI parameters for the S<sub>2</sub> state, (iii) provides the first reliable HFI tensors for the S<sub>0</sub> state, and (iv) leads to a suggestion for the Mn oxidation states in S<sub>0</sub> and S<sub>2</sub>.

PSII membranes were prepared according to standard procedures<sup>8</sup> and washed several times after the Triton treatment to completely remove starch. S<sub>2</sub> state samples were obtained by concentrating dark-adapted PSII membranes containing 250 μM PPBQ (phenyl-*p*-benzoquinone) and 1 mM EDTA in 3 mm quartz tubes by centrifugation (~25 mg of chlorophyll/mL final) and subsequent 200 K illumination. S<sub>0</sub> state samples were prepared using the three-flash/FCCP approach described previously.<sup>8,12</sup> The samples were finally concentrated by centrifugation in the Q-band EPR tubes. After completion of the <sup>55</sup>Mn ENDOR experiments, the S<sub>0</sub> state population was determined to be 65 ± 5% (the rest being S<sub>1</sub>) on the basis of the amplitude of the S<sub>2</sub> EPR multiline signal generated by 200 K illumination compared to that of S<sub>2</sub> control samples. All samples contained 3% (v/v) methanol.

The experiments were performed on a Bruker ESP-580 Q-band pulse EPR spectrometer equipped with a home-built ENDOR cavity. The pulse ENDOR experiments were performed using SpecMan



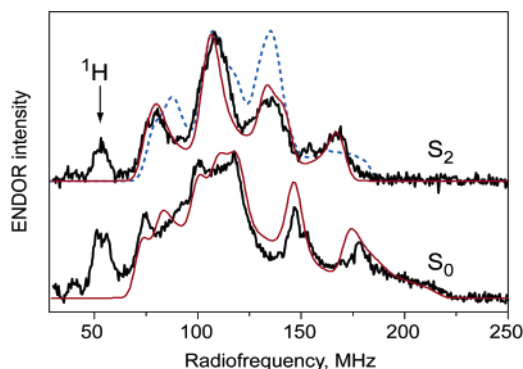
**Figure 1.** Q-band echo-detected EPR spectra (light minus dark) of the S<sub>2</sub> and S<sub>0</sub> states (A) and their numerical pseudomodulation with 20 G amplitude, which gives CW EPR-like derivative spectra (B). *T* = 4.5 K, mw  $\pi/2$ -pulse 32 ns,  $\tau$  = 260 ns,  $\nu_{mw}$  = 33.850 GHz (S<sub>2</sub> state), 34.123 GHz (S<sub>0</sub> state). The spectra of the S<sub>2</sub> state are shifted by 100 G to higher magnetic field to compensate for the mw frequency difference. The pulse repetition time was 1 ms (S<sub>2</sub> state) and 12 μs (S<sub>0</sub> state). The arrows indicate the magnetic field positions where <sup>55</sup>Mn ENDOR spectra were taken (see Figure 2). In the S<sub>2</sub> state spectra, the sharp signal of the tyrosine D radical of PSII at *g* ≈ 2 is removed for clarity. This signal is practically absent for the S<sub>0</sub> state sample due to chemical reduction by FCCP.

control software that varies the radio frequency (rf) randomly in the desired range. This leads to a decrease of the rf-induced heat accumulation in the resonator and in turn to a reduction of heating artifacts.

Figure 1A shows the two-pulse echo-detected EPR spectra of the S<sub>2</sub> and S<sub>0</sub> states (light minus dark difference). A comparison reveals that the S<sub>0</sub> signal is ~500 G broader and that the extra width mainly stems from the high-field wing. This indicates substantial *g*-anisotropy in the S<sub>0</sub> state and shows that *g*<sub>iso</sub> of the S<sub>0</sub> state is smaller than *g*<sub>iso</sub> of the S<sub>2</sub> state. The CW EPR-like derivative spectra of Figure 1B are produced by numerical pseudomodulation of the field sweeps (Figure 1A) using a 20 G amplitude. This procedure allows a closer inspection of the hyperfine structure. For the S<sub>0</sub> state, this is the first report of a CW-like Q-band EPR spectrum. The total width is about 2500 G, and at least 26 lines are resolved. At the low-field side the average splitting is about 78 G, and at the high-field side it is 84 G. The overall similarity to the well-known S<sub>0</sub> state CW EPR X-band spectrum confirms that the Q-band two-pulse echo originates from the S<sub>0</sub> state. An inversion–recovery measurement of the spin lattice relaxation time *T*<sub>1</sub> for the S<sub>0</sub> state (data not shown) gives *T*<sub>1</sub> ≈ 9 μs at 4.5 K, which is about 100 times faster than the *T*<sub>1</sub> of the S<sub>2</sub> state.<sup>13</sup> This is in line with previous reports that the S<sub>0</sub> state has a higher half-saturation power than the S<sub>2</sub> state.<sup>9,14</sup>

The black lines in Figure 2 show the <sup>55</sup>Mn ENDOR spectra of the S<sub>2</sub> and S<sub>0</sub> states that were recorded at the magnetic field

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**Figure 2.**  $^{55}\text{Mn}$  pulse ENDOR spectra at Q-band of the  $S_2$  and  $S_0$  states (black) and simulations using either the parameters of Table 1 (red) or those determined previously by Pelouin et al.<sup>11</sup> on the basis of X-band  $^{55}\text{Mn}$  ENDOR (broken blue line). The Davies ENDOR sequence was used with a radio frequency (rf)  $\pi$ -pulse duration of 5  $\mu\text{s}$  ( $S_2$  state) and 4  $\mu\text{s}$  ( $S_0$  state). Temperature and other conditions are the same as in Figure 1.  $B_0 = 12600$  G ( $S_2$  state) and 12550 G ( $S_0$  state).

**Table 1.** Principal Values of HFI Tensors (Absolute Values) of Individual  $^{55}\text{Mn}$  Ions Used for Simulation of the  $^{55}\text{Mn}$  ENDOR Spectra of the OEC

	$S_2$ state		$S_0$ state	
	$A_{1i}$ , MHz	$A_{1b}$ , MHz	$A_{1i}$ , MHz	$A_{1b}$ , MHz
$\text{Mn}_A$	235	265	270	200
$\text{Mn}_B$	185	245	190	280
$\text{Mn}_C$	310	265	320	400
$\text{Mn}_D$	175	230	170	240

positions indicated by the arrows in Figure 1A. Similar results were obtained at other field positions, which indicates a negligible orientation selection (data not shown). The  $S_2$  state spectrum spans from 70 to 180 MHz, which is 20 MHz wider than at X-band. This difference is readily explained by the increased Zeeman frequency of the Mn nuclei (13 MHz at Q-band vs 3 MHz at X-band). The spectrum of the  $S_0$  state is broader than that of the  $S_2$  state; a weak shoulder extends to 220 MHz. Its low-frequency edge is probably at 70 MHz, but there is the possibility that a weak wing spans below the proton peak down to  $\sim 50$  MHz. Scans up to 400 MHz do not reveal any additional signals.

Because of the small orientation selection, the mean  $^{55}\text{Mn}$  ENDOR frequency (the “center of gravity” of the spectrum) equals approximately half of the absolute value of the isotropic Mn HFI constants averaged over the four Mn nuclei,  $\langle |A_{\text{iso}}| \rangle / 2$ . For theoretical spectra, this procedure has shown a precision of better than 1%. Analysis of the experimental  $^{55}\text{Mn}$  ENDOR spectrum of the  $S_0$  state gives  $\langle |A_{\text{iso}}| \rangle = 250$  MHz. In the case of isotropic Mn HFI tensors, their contribution to the width of the EPR spectrum is  $W_{\text{HFI}} = 5N\langle |A_{\text{iso}}| \rangle$ , where  $N$  is the number of Mn nuclei. Any anisotropy in the HFI increases this value. The simulation shows that this increase is about 12% for the  $S_0$  state (Table 1). The total width of the X-band  $S_0$  state EPR spectrum is  $W_T = 6160\text{--}6700$  MHz ( $g = 2$ ).<sup>8</sup> With  $N = 4$ , we obtain  $W_{\text{HFI}} = 5600$  MHz. The difference between  $W_{\text{HFI}}$  and  $W_T$  can be explained by a  $g$ -tensor anisotropy of  $\Delta g \approx 0.1\text{--}0.2$ , which is in the expected range. A trinuclear or binuclear origin for the  $S_0$  multiline requires a much larger  $g$ -tensor anisotropy of  $\Delta g \approx 0.4\text{--}0.5$  or  $0.65\text{--}0.75$ , respectively. This strongly disfavors such proposals. For the  $S_2$  state, the comparison of  $W_T = 5400$  MHz and  $W_{\text{HFI}} = 4700$  MHz (Q-band) gives an

upper limit of  $\Delta g \approx 0.04$  for  $N = 4$ , which agrees with the previously reported values of  $\Delta g \approx 0.02\text{--}0.04$ .<sup>11,15</sup> and further supports the idea that all four Mn nuclei are magnetically coupled in both states.

The red lines in Figure 2 show numerical simulations of the  $^{55}\text{Mn}$  ENDOR spectra, performed using second-order perturbation theory.<sup>16</sup> Only the allowed transitions were taken into account. For simplicity, axial  $g$ -tensors and Mn HFI tensors with coinciding directions for the symmetry axes were assumed, and Mn nuclear quadrupole interaction (NQI) was excluded (Table 1). Within this simple model, the precision of the simulation is  $\pm 10$  MHz. Since the signs of HFI constants cannot be determined by ENDOR simulations, only their absolute values are presented. We used  $g_{\perp} = 1.97$ ,  $g_{\parallel} = 1.99$  for the  $S_2$  state and  $g_{\perp} = 1.99$ ,  $g_{\parallel} = 1.89$  for the  $S_0$  state (see above). However, the simulated  $^{55}\text{Mn}$  ENDOR spectrum is essentially insensitive to the variation of the principal values of the  $g$ -tensor. The HFI parameters for the  $S_2$  state are close to those derived previously on the basis of X-band  $^{55}\text{Mn}$  ENDOR; a simulation using these previously reported parameters is shown for visual comparison as the broken blue line in Figure 2.<sup>11</sup>

Overall, surprisingly similar  $^{55}\text{Mn}$  ENDOR spectra (Figure 2) and HFI parameters (Table 1) are found in this study for the  $S_0$  and  $S_2$  states. This appears to favor  $\text{Mn}_4(\text{III}, \text{III}, \text{III}, \text{IV})$  and  $\text{Mn}_4(\text{III}, \text{IV}, \text{IV}, \text{IV})$  as oxidation states for the  $S_0$  and  $S_2$  states, respectively, over alternative assignments that contain one Mn(II) in the  $S_0$  state. This idea and possible structural models for the  $\text{Mn}_4\text{O}_x\text{Ca}$  complex will be tested in a forthcoming publication, where the obtained HFI parameters will be used together with various spin-coupling schemes for the simulation of the hyperfine structures of the  $S_2$  and  $S_0$  EPR signals.

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## References

- Messinger, J. *Phys. Chem. Chem. Phys.* **2004**, *6*, 4764–4771.
- Carell, T. G.; Tyryshkin, A. M.; Dismukes, G. C. *J. Biol. Inorg. Chem.* **2002**, *7*, 2–22.
- McEvoy, J. P.; Brudvig, G. W. *Phys. Chem. Chem. Phys.* **2004**, *6*, 4754–4763.
- Ferreira, K. N.; Iverson, T. M.; Maghlaoui, K.; Barber, J.; Iwata, S. *Science* **2004**, *303*, 1831–1838.
- Biesiadka, J.; Loll, B.; Kern, J.; Irrgang, K.-D.; Zouni, A. *Phys. Chem. Chem. Phys.* **2004**, *6*, 4733–4736.
- Kok, B.; Forbush, B.; McGloin, M. *Photochem. Photobiol.* **1970**, *11*, 457–476.
- Dismukes, G. C.; Siederer, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 274–278.
- Messinger, J.; Robblee, J. H.; Yu, W. O.; Sauer, K.; Yachandra, V. K.; Klein, M. P. *J. Am. Chem. Soc.* **1997**, *119*, 11349–11350.
- Messinger, J.; Nugent, J. H. A.; Evans, M. C. W. *Biochemistry* **1997**, *36*, 11055–11060.
- Ahrling, K. A.; Peterson, S.; Styring, S. *Biochemistry* **1997**, *36*, 13148–13152.
- Peloquin, J. M.; Campbell, K. A.; Randall, D. W.; Evanchik, M. A.; Pecoraro, V. L.; Armstrong, W. H.; Britt, R. D. *J. Am. Chem. Soc.* **2000**, *122*, 10926–10942.
- Robblee, J. H.; Messinger, J.; Cinco, R. M.; McFarlane, K. L.; Fernandez, C.; Pizarro, S. A.; Sauer, K.; Yachandra, V. K. *J. Am. Chem. Soc.* **2002**, *124*, 7459–7471.
- Lorigan, G. A.; Britt, R. D. *Biochemistry* **1994**, *33*, 12072–12076.
- Peterson, S.; Ahrling, K. A.; Styring, S. *Biochemistry* **1999**, *38*, 15223–15230.
- Zheng, M.; Dismukes, G. C. *Inorg. Chem.* **1996**, *35*, 3307–3319.
- Sturgeon, B. E.; Ball, J. A.; Randall, D. W.; Britt, R. D. *J. Phys. Chem.* **1994**, *98*, 12871–12883.

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