Parallel Polarization Electron Paramagnetic Resonance Studies of the S_1-State Manganese Cluster in the Photosynthetic Oxygen-Evolving System†

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ABSTRACT: Magnetic properties of the S_1-state manganese cluster in the oxygen-evolving photosystem II were studied by parallel polarization electron paramagnetic resonance spectroscopy. Dark minus light spectra gave rise to a broad S_1-state signal with a g value of about 4.9 [Dexheimer, S. L., Klein, M. P. (1992) *J. Am. Chem. Soc. 114*, 2821−2826]. Temperature variation of the signal intensity between 1.9 and 10 K observed in PS II with a sucrose buffer indicates that the signal originates from an excited state with a spin S of 1 with separation from the ground state ($S = 0$) of about 2.5 K. The S_1-state signal was also observed in the sucrose buffer supplemented by 50% glycerol. However, no S_1-state signal was detected by addition of 3% methanol or 30% ethylene glycol in the sucrose buffer, although illumination at 200 K in the presence of these alcohols induced the normal multiline S_2 signal. Furthermore, modification of the Mn cluster by Cl^- or Ca^{2+} depletion from PS II membranes failed to produce a detectable S_1-state signal. A possible magnetic structure of the Mn cluster responsible for the generation of the S_1-state signal is discussed on the basis of these observations.

Oxygen evolution in plant photosynthesis is accomplished by a protein complex called photosystem II (PS II).[1] An oxygen-evolving complex located at the lumen side of the PS II complex contains a tetranuclear Mn cluster which is believed to accumulate positive charges and to provide a catalytic site for water oxidation. The driving force for the generation of oxidizing equivalents is supplied by the excitation of a primary electron donor of PS II (P680) and the successive charge separation into $P680^+Q_A^-$. The oxidized P680 is subsequently re-reduced by delivery of an electron from the Mn cluster via the redox active tyrosine residue (Y_Z) of the D1 protein. Illumination of PS II results in successive abstraction of four electrons from the OEC and the evolution of molecular oxygen (Kok et al., 1970). The process involves five intermediate oxidation states labeled S_i ($i = 0-4$) in which S_1 is most stable in the dark. By absorbing each photon, the S_0 or S_1 state advances stepwise to reach the highest oxidation state S_4, which spontaneously decays to the S_0 state with concurrent release of molecular oxygen [reviewed in Rutherford et al. (1992) and Debus (1992)]. X-ray absorption near edge structure (XANES) studies indicated that the S-state transitions involve changes in the valence state of the Mn cluster (Ono et al., 1992; Roelofs et al., 1996).

EPR spectroscopy has been applied extensively to the study of the Mn cluster in the OEC in order to provide information regarding its magnetic structure. By conventional X-band EPR measurement, S_2 is the only state giving rise to two types of EPR signals. The first signal is centered at $g = 2$ with an overall line width of approximately 1600 G and is denoted as the multiline signal (Dismukes & Siderer, 1981). The other is centered at $g = 4.1$ with a peak-to-trough line width of approximately 400 G (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984; de Paula et al., 1985, 1987). The multiline signal exhibits 18−20 partially resolved hyperfine lines that are spaced by 85−90 G [reviewed in Debus (1992)]. The signal is ascribed to the ground state with an S of $^1/_2$ (Pace et al., 1991; Britt et al., 1992). Detailed spectral simulations of this signal suggest that the manganese cluster is a multinuclear complex including a Mn(III)−Mn(IV) pair (Årling & Pace, 1995; Randall et al., 1995). Upon illumination of samples at 140 K, the $g = 4.1$ signal was induced preferentially to the multiline (Casey & Sauer, 1984; de Paula et al., 1985, 1987). This signal thus formed converts to the multiline signal when annealed at 200 K in the dark. The two signals are generated concurrently upon illumination at 200 K in the presence of sucrose (Zimmermann & Rutherford, 1984). The $g = 4.1$ signal has been assigned to arise from the Mn cluster having a $^5/_2$ spin state (Astashkin et al., 1994; Haddy et al., 1992) or a $^3/_2$ spin state (Smith et al., 1993, 1996; Hansson et al., 1987). The presence of various alcohols with low molecular weights (glycerol, ethylene glycol, ethanol, and methanol) suppresses the formation of the $g = 4.1$ signal upon illumination at 200 K and complementarily enhances the multiline signal intensity (Zimmermann & Rutherford, 1986; Pace et al., 1991).

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[1] Abbreviations: PS II, photosystem II; OEC, oxygen-evolving complex; Chl, chlorophyll; P680, primary donor of PS II; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; XANES, X-ray absorption near edge structure; Y_Z, redox active tyrosine 161 of the D1 protein; Y_D, redox active tyrosine 161 of the D2 protein.

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Pace et al. (1991) have studied the temperature variation of the characteristics of the multiline and $g = 4.1$ signals in the presence or absence of 3% methanol and suggested that the exchange coupling between two dimers of manganese was modified by alcohol.

The multiline and the $g = 4.1$ signal characteristics have been modified by depletion of either Ca^{2+} or Cl^-, which are indispensable cofactors for oxygen evolution. On the basis of changes in the relative distances between manganese atoms determined by extended X-ray absorption fine structure (EXAFS), it has been suggested that depletion of these cofactors causes a structural modification of the Mn cluster (Liang et al., 1994; Dau et al., 1995).

In the S_1 state, there had been no observed signal, before the parallel polarization EPR was applied to this state by Dexheimer and Klein (1992). As the S_2 state has a half-integer spin of $^1/_2$, the S_1 state is considered to have an integer spin. Because of a weak relaxation effect on the tyrosine D radical (Koulougliotis et al., 1992; Un et al., 1995) and a weak paramagnetic susceptibility (Babcock et al., 1990), the S_1 state has been suggested to be a diamagnetic state. However, we could detect the relaxation effect in a selective hole-burning experiment on the tyrosine D radical in the S_1 state and successfully apply $S = 1$ to determine the distance between the S_1-state Mn cluster and tyrosine D (Kodera et al., 1994).

Parallel polarization EPR studies have been performed to detect $\Delta m_S = 0$ transitions connected with spin matrix S_Z, which has been often called the "forbidden transition" for a triplet state [see Carrington and Mclachlan, (1967)]. Since the line width is narrower in forbidden than in allowed transitions, it is possible to observe a broad signal which cannot be detected in an allowed transition. This method is furthermore useful for the determination of zero-field splitting parameters D and E. Dexheimer and Klein (1992) have applied this technique to the OEC in PS II and observed a new EPR signal arising from the Mn cluster in the S_1 state. The signal centered at $g = 4.8$ with a peak-to-trough width of about 600 G has been attributed to the $S = 1$ spin state of a weakly antiferromagnetic-coupled manganese dimer (Dexhimer & Klein, 1992). The decrease of the S_1-state signal intensity demonstrated a linear relation to the increase of the S_2-state multiline signal intensity upon illumination, while no correlation was observed between the intensities of the S_1-state and $g = 4.1$ signals. From the above observations, it has been concluded that the S_1-state signal arises from the manganese center responsible for the multiline signal rather than from the center that reveals the $g = 4.1$ signal. Despite much effort, reproduction of the S_1-state signal has not been successful since its first discovery (Brudvig, 1995).

In this report, we have studied the S_1-state Mn cluster by means of parallel polarization EPR. In order to not only confirm the S_1-state signal but also obtain more information about the signal characteristics, the effect of temperature variation on the signal characteristics has been observed. The signals have also been investigated in alcohol-supplemented and inhibited preparations of PS II. Indeed, results obtained have confirmed that the S_1 state reveals the EPR signal at about $g = 4.9$ with a line width of 600 G. The temperature dependence of the signal intensity indicates that the signal may be attributed to an excited state of $S = 1$ separated from the ground state ($S = 0$) by approximately 2.5 K. On the basis of these results, a possible magnetic interaction between the Mn atoms responsible for the S_1-state signal is discussed.

MATERIALS AND METHODS

Oxygen-evolving PS II membranes were prepared from market spinach by the method of Kuwabara and Murata (1982) or Berthold et al. (1981) with modification (Ono & Inoue, 1986) and stored in liquid N_2 until use. No differences in the EPR spectral characteristics were detected between these preparations. The following procedures were conducted in complete darkness or under a dim green safe light unless otherwise noted. Thawed membranes were suspended in a buffer medium containing 0.4 M sucrose, 14 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM Mes/NaOH (pH 6.0) or a medium supplemented with 30% ethylene glycol, 50% glycerol or 3% methyl alcohol after two washes with each medium. Sucrose (0.2 M) was used for ethylene glycol or glycerol-containing buffer medium instead of 0.4 M sucrose.

For Cl^- depletion, the membranes were suspended in the medium containing 0.4 M sucrose, 50 mM Na_2SO_4, and 0.5 mM EDTA, and 40 mM Hepes/NaOH (pH 7.5), after two washes with the same medium as described in Ono et al. (1986). Ca^{2+} depletion was carried out by low-pH treatment as described in Ono and Inoue (1992), and the resulting membranes were suspended in a medium containing 0.4 M sucrose, 20 mM NaCl, and 40 mM Mes/NaOH (pH 6.5). DCMU (50 mM, 0.5% of a 10 mM stock solution of dimethyl sulfoxide) was added into the final buffer in Cl^-- and Ca^{2+}-depleted membranes to ensure a single turnover of the PS II reaction center. Mn removal was performed by washing of the membranes with 0.8 M Tris-HCl at pH 8.0 and 273 K for 30 min under room light at a concentration of 0.3 mg of Chl/mL (Kodera et al., 1995). After one wash, the pellet was suspended in the same pH 6.0 buffer as that before Tris washing. Samples with a chlorophyll concentration of about 15 mg of Chl/mL were transferred into Suprasil quartz tubes with 4 mm inner diameter.

EPR samples except for Ca^{2+}- and Cl^--depleted PS II were preilluminated for 6 min at 200 K with a 500 W tungsten bromide lamp through a 10 cm thick water filter and then dark adapted for 1 h at 273 K to ensure 100% S_1 state (Styring & Rutherford, 1988). For Ca^{2+}-depleted PS II, the samples were illuminated at 273 K for 1 min, 200 K for 6 min, or 140 K for 10 min in a temperature-controlled ethanol or isopentane/ethyl ether bath after measurements of dark-state signals. In each measurement, the background signal appearing in the illuminated sample or the dark-adapted sample is subtracted. As we found that background signal intensities are strong at the sealed bottom of sample tubes, we hold this part 20 mm lower than the center of the cavity.

EPR measurements were carried out on a Bruker ESP 300E X-band spectrometer equipped with an ER4116 DM X-band dual-mode resonator. The microwave frequency was changed in order to resonate with perpendicular TE_{102} or parallel TE_{012} mode. Oxford 900 and 910 continuous flow cryostats were used for the measurements above and below 4.2 K, respectively. The sample temperature indicated by a gold−iron (0.07 at. %) chromel thermocouple was calibrated by a calibrated carbon−glass resistor inserted into a sample tube containing a buffer medium. For a lower temperature below 4.2 K, the temperature was read from the vapor

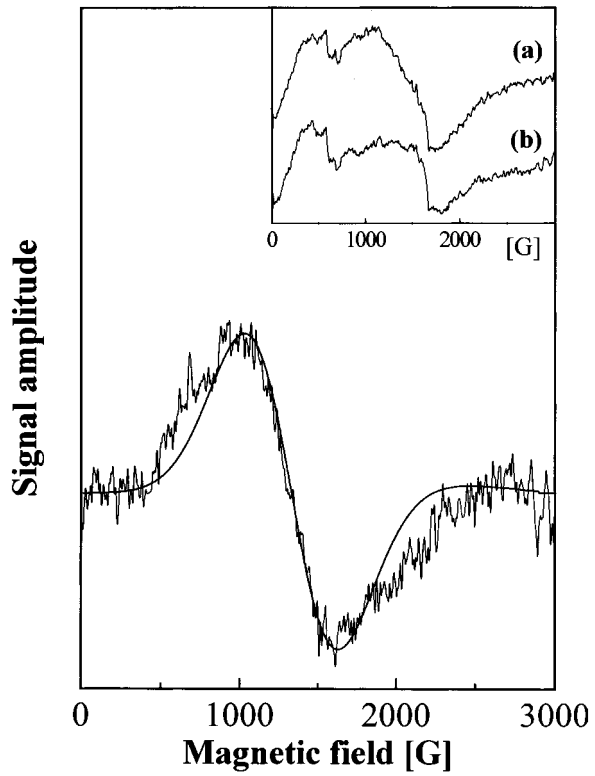

FIGURE 1: Parallel polarization EPR signal for the S_1 state in untreated PS II membranes. The signal was obtained by subtracting the 200 K illuminated spectrum from the dark-adapted spectrum. The inset shows parallel polarization EPR spectra from (a) dark-adapted and (b) 200 K illuminated membranes. PS II membranes were suspended in 0.4 M sucrose buffer. EPR conditions: microwave frequency, 9.34 GHz; microwave power, 3.2 mW; field modulation amplitude, 8 G at 100 kHz; scan time, 168 s; time constant, 0.2 s; and temperature, 5 K. Twenty scans were accumulated before and after illumination. The solid line displays the result of computer simulation using the following simulation parameters: $S = 1$, $g = 2.0$, $D = -0.140$ cm^{-1}, and $E/D = -0.25$. See the text for details.

pressure inside the cryostat when liquid helium was pumped. The value of the vapor pressure has been also calibrated by the carbon−glass resistor in the sample tube. At 4.8 K, higher- and lower-temperature data were connected. The temperature dependence of the intensity of the Y_D^+ signal was measured as an internal standard in the perpendicular mode to confirm the accuracy of temperature measurements before measurements in the parallel polarization mode. An external AFC (automatic frequency control) was applied to detect the Y_D^+ signal at low microwave powers below 1 μW. As the temperature dependence of the Y_D^+ signal intensity followed a Curie law, the change in cavity Q could be neglected in the studied temperature range.

RESULTS AND DISCUSSION

Figure 1 shows the parallel polarization EPR spectra of the S_1 state (dark) minus the S_2 state (light) observed at 5 K in untreated PS II membranes. The difference spectrum shows a broad featureless signal at $g = 4.9 \pm 0.1$, with a line width of about 600 G. These values are in good agreement with those of the S_1-state signal originally reported by Dexheimer and Klein (1992). Only a dark minus light difference spectrum (c) reveals the S_1-state signal, because large and overlapping background signals are always present, as shown in the inset of Figure 1. The background signals

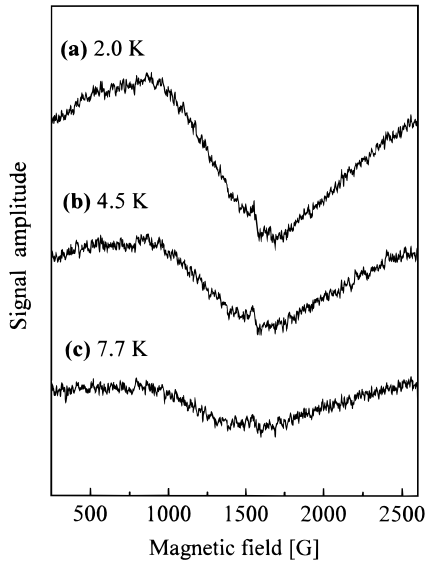

FIGURE 2: Effect of temperature on the parallel polarization EPR signal for the S_1 state in untreated PS II membranes. Spectra were measured at 2.0 K (a), 4.5 K (b), and 7.7 K (c), respectively. PS II membranes were suspended in 0.4 M sucrose-supplemented buffer. EPR conditions are the same as those in Figure 1.

were observed for empty tubes and could therefore be considered to be due to impurities in the sample tubes. The S_1-state signal intensity at 5 K was estimated to be about 40 $\pm$ 10% of that of the Y_D^+ signal from comparison of values obtained by double integration of these two signals over each magnetic field range.

For simulation of the S_1-state signal, the spin Hamiltonian for a $S > {}^1/_2$ state is given by

$$H = \beta \mathbf{H}_0 \cdot \mathbf{g} \cdot \mathbf{S} + D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) \quad (1)$$

where D and E are zero-field splitting parameters and **g** and β are the **g** tensor and Bohr magneton, respectively. As the line shape of the S_1-state signal is rather symmetric with respect to the base line, $S = 1$ and an isotropic g value of 2.0 are assumed. In order to determine the transition probability for the parallel polarization mode at a given resonance frequency of 9.38 GHz, diagonalization of eq 1 was carried out, for various values of input parameters D, E, H_0, and the magnetic field direction (θ, φ) relative to the zero-field z-axis of the manganese cluster. The E/D ratio ranges between 0 in an axial to $\pm$0.33 in a completely rhombic crystalline field. The transition probabilities were then calculated using the determined energy levels and wave functions. After calculation of the powder pattern using a Gaussian line broadening with $\Delta\nu = 1400$ MHz, a relatively satisfactory fit was obtained with parameter values $D = -0.140$ cm^{-1} and $E/D = -0.25$, from the allowable ranges -0.12 cm$^{-1} > D > -0.15$ cm^{-1} and $-0.15 > E/D > -0.33$, respectively. These values are almost coincident with those reported previously by Dexheimer and Klein (1992) within the experimental error. Figure 1 also shows the resultant simulation fitted to the observed S_1-state spectrum (solid line). The discrepancy at both wings of the spectrum may be ascribed to a difference of the actual line shape from the assumed Gaussian.

Figure 2 shows the S_1-state signals measured at three different temperatures. With increasing temperature, the signal intensity decreases without a change in the line shape.

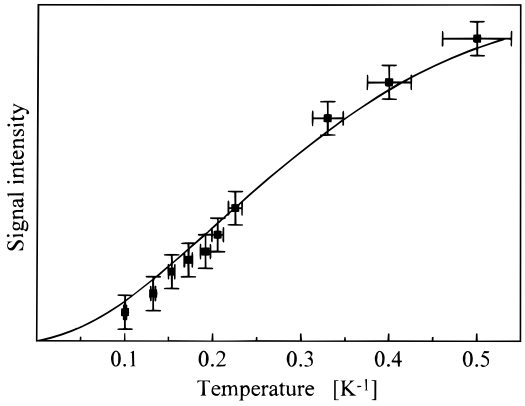

FIGURE 3: Temperature dependence of the S_1-state signal intensity in untreated PS II membranes. The signal intensity was estimated as the trough-to-peak height at approximately $g = 4.9$. Vertical and horizontal bars indicate error ranges for estimation of the signal intensity and temperature, respectively. The curve is a fit to eq 4 in the text, where $S = 1$, $J = -0.87$ cm^{-1}, and summation of j was taken over five spin states of the Mn(III)−Mn(III) dimer.

The signal almost disappears below the noise level above 10 K. Figure 3 shows the temperature dependence of the S_1-state signal intensity, where the trough-to-peak height is plotted against the inverse temperature. The plot follows a Curie law above 5 K and slightly deviates to lower values below 4 K. Measurements at lower microwave powers confirmed that the S_1-state signal observed at 3.2 mW was not saturated at every temperature.

To simulate the obtained temperature dependence with non-Curie law behavior, a system with two exchange-coupled spins, S_1 and S_2, is assumed for simplicity. The spin Hamiltonian for such exchange coupling is given by

$$H = -2J\mathbf{S}_1\cdot\mathbf{S}_2 \quad (2)$$

The energy levels are then given for the coupled spin $\mathbf{S} = \mathbf{S}_1 + \mathbf{S}_2$, neglecting the contribution from Zeeman and crystalline field interactions (see eq 1).

$$E_S = -J[S(S+1) - S_1(S_1+1) - S_2(S_2+1)] \quad (3)$$

where S takes a spin number between $|S_1 - S_2|$ and $S_1 + S_2$. The signal intensity for the spin state with S at an arbitrary temperature T is proportional to its Boltzmann population and is given by

$$I(T) = (I_0/T)(2S+1)\exp(-E_S/kT)/\sum_j(2S_j+1) \times \exp(-E_j/kT) \quad (4)$$

where I_0 is a constant to be fitted. If we assume an antiferromagnetically coupled dimer, Mn(III)−Mn(III), the maximum value of $S = S_1 + S_2$ is 4 and summation on j was taken over five states with S ranging from 0 to 4. The signal intensity for the first excited state $S = 1$, calculated from eq 4, could be best fitted to the observed temperature dependence of the S_1-state signal using a J value of -0.87 cm^{-1}. For other cases, Mn(IV)−Mn(IV) and Mn(II)−Mn(II) dimers could be taken into consideration. Then the maximum values of S are 3 and 5, respectively, in eq 4. However, the obtained J value was insensitive to the assumed spin number, as far as the temperature dependence of the population for the $S = 1$ sublevel (the first excited state) was concerned. The S_1-state signal can, therefore, be assigned to a first excited state of a weakly antiferromagnetically coupled dimer with both centers having the same spin value. The interpretation could also be extended to the model involving a dimer of two manganese dimers, by assuming each dimer has the same net spin value and that they are coupled to a resultant zero spin in the ground state by a magnetic exchange interaction. Therefore, the obtained J value would be compatible with various models of the Mn cluster presented so far (Smith et al., 1996; Kusunoki, 1992; Chan & Armstrong, 1991).

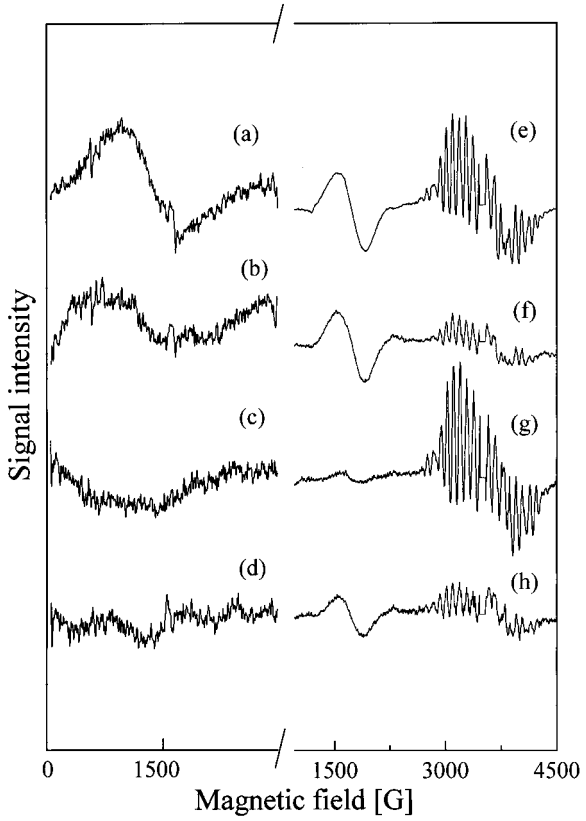

FIGURE 4: Effect of methanol on EPR signals in untreated membranes. EPR signals were observed in the dark-adapted minus illuminated difference spectrum for the parallel polarization mode (a−d) and the illuminated minus dark-adapted spectrum for the conventional perpendicular polarization mode (e−h). Membranes were illuminated at 200 K for 6 min (a, c, e, and g) or illuminated at 140 K for 10 min (b, d, f, and h) in 0.4 M sucrose (a, b, e, and f) or 3% methanol-supplemented (c, d, g, and h) buffer, respectively. EPR conditions for conventional perpendicular polarization mode: microwave frequency of 9.64 GHz and four scans accumulated. Other conditions are as in Figure 1.

Taking the Boltzmann population of 0.41 estimated at 5 K from eq 4 using $J = -0.87$ cm^{-1} and the field-frequency conversion factor of about 1.6 for the obtained D and E values into consideration, the simulation of signal intensities for $S = 1$ and $S = {}^1/_2$ states showed that the experimental relative intensity of the S_1 state to Y_D^+ signal ($40 \pm 10\%$) corresponds to $75 \pm 25\%$ of the S_1 state that produced the Y_D^+ signal.

Figure 4 shows the effects of methanol on the formation of the S_1-state EPR signal. Upon illumination of the PS II membranes in 0.4 M sucrose buffer at 200 K, the S_1-state signal was clearly observed by subtraction of the S_2-state background (trace a), and both the multiline and the $g = 4.1$ signal obtained in the conventional mode were obtained (trace e). Illumination at 140 K preferentially induced the $g = 4.1$ signal (trace f), although the total yield of S_2-state formation was lower compared to that with 200 K illumina-

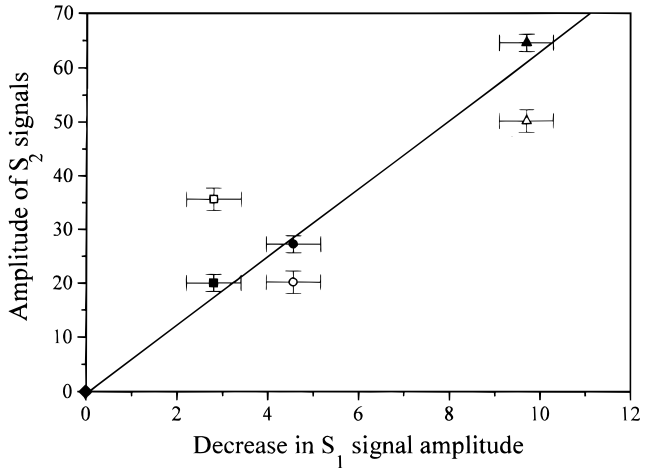

FIGURE 5: Relationship between S_1-state and S_2-state EPR signals in untreated PS II membranes. The S_1-state signal was observed in the dark-adapted minus illuminated difference spectrum for the parallel polarization mode, and multiline (closed symbols) and $g = 4.1$ (open symbols) S_2-state signals were observed in the illuminated minus dark-adapted spectrum for the conventional perpendicular polarization mode. Signal intensities were determined as the trough-to-peak height for the S_1-state signal and the $g = 4.1$ signal and as the sum of the trough-to-peak heights of the fourth and fifth lines at the lower field side from the Y_D^+ signal for the multiline signal. Sample membranes were illuminated at 140 K for 10 min (squares), followed by dark adaptation at 200 K for 1.5 min (circles), and then illuminated at 200 K for 6 min (triangles). Bars indicate experimental errors. EPR conditions are the same as those in Figure 1 for parallel polarization and the same as those in Figure 4 for the perpendicular polarization mode. The 100% decrease in the S_1-state signal intensity corresponds to the value of 11 on the *x*-axis, and the 100% multiline intensity corresponds to the value of 70 along the *y*-axis.

tion, due to oxidation of the high-potential cytochrome b_{559} in competition with the Mn cluster. The intensities of the multiline and $g = 4.1$ signals were about 30 and 70%, respectively, of those induced by illumination at 200 K. The S_1-state signal intensity decreased by 140 K illumination was approximately 30% of that observed by 200 K illumination (trace b), matching the lower multiline formation (trace f).

Experiments performed using the buffer supplemented by 3% methanol, however, produced results remarkably different from those obtained by using the buffer without methanol. The S_1-state signal (dark minus light spectra) was not detected upon illumination at either 200 (trace c) or 140 K (trace d). The multiline signal, however, was normally induced in the absence of the $g = 4.1$ signal upon illumination at 200 K (trace g), while the $g = 4.1$ signal was preferentially induced upon illumination at 140 K (trace h).

It has been reported that the decrease in the intensity of the S_1-state signal upon illumination is quantitatively related to the increase in the intensity of the multiline signal but not related to that of the $g = 4.1$ signal (Dexheimer & Klein, 1992). The results have been interpreted to mean that the oxygen-evolving center giving rise to the S_1-state signal converts only to the center responsible for the multiline signal. After the 140 K illuminated sample was annealed (Figure 4f) at 200 K for about 1.5 min, the intensity of the multiline signal increased. As shown in Figure 5, there is a distinct linear relation between the increase in multiline and the decrease in S_1-state signals in membranes suspended in the buffer containing 0.4 M sucrose. However, no correlation was observed between the $g = 4.1$ and the S_1-state signals.

Table 1: Effects of Various Treatments and Illumination Temperatures on Mn EPR Signals Generated in PS II Membranes

	S_1-state signal			multiline signal			$g = 4.1$ signal		
samples	140[f]	200[f]	273[f]	140[f]	200[f]	273[f]	140[f]	200[f]	273[f]
untreted PS II									
+0.4 M sucrose	+[a]	++[b]	nd[c]	+	++	nd	++	++	nd
+50% glycerol	+	++	nd	+	++	nd	++	+	nd
+30% ethylene glycol	−[d]	−	nd	+	++	nd	++	−	nd
+3% methanol	−	−	nd	+	++	nd	++	−	nd
Cl^--depleted PS II	nd	−	−	nd	−	−	nd	++	++
Ca^{2+}-depleted PS II	nd	nd	−	nd	nd	++m[e]	nd	nd	−
Tris-treated PS II	nd	−	−	nd	−	−	nd	−	−

[a] Less than 40% of the intensity of the maximum of the corresponding difference spectrum observed in untreated PS II with sucrose buffer. [b] More than 50% of the intensity of the maximum of the corresponding difference spectrum observed in untreated PS II with sucrose buffer. [c] No data. [d] No signal. [e] Modified signal. The Intensity of the S_1-state signal was evaluated by generating a dark minus illuminated spectrum. [f] Illumination temperature in kelvin.

A similar relationship between the S_1-state and S_2-multiline signals was observed using a 50% glycerol-supplemented buffer (data not shown), which has been used by Dexheimer and Klein (1992).

Table 1 summarizes effects of illumination temperature and various preparations on generation of the S_1-state, multiline, and $g = 4.1$ signals. The results indicate that the capacity to produce the S_1-state signal is markedly affected by the presence of methanol and ethylene glycol, or inhibitory treatments (Cl^- and Ca^{2+} depletion). No S_1-state signal was detected in the presence of 30% ethylene glycol or 3% methanol, although illumination at 200 K induced the normal multiline signal. The results, therefore, imply that the formation of the multiline signal is not always accompanied by a decrease of the S_1-state signal. It has been reported that low-molecular weight alcohols suppress the formation of the $g = 4.1$ signal in samples illuminated above 200 K (Zimmermann & Rutherford, 1986). The effect of alcohol, however, is not attributable to the inhibition of the S_1 state, since the multiline signal is normal and even pronounced in the presence of 3% methanol. The S_1-state signal was not detected in the Ca^{2+}- and Cl^--depleted membranes in which the Mn cluster has been considered to be structurally modified (Liang et al., 1994). As Cl^--depleted membranes produce a relatively strong $g = 4.1$ signal upon illumination at 200 K, it is of note that the presence of the S_1-state signal is not correlated to the formation of the $g = 4.1$ signal in both untreated and inhibited preparations.

The present results show that the formation of both the multiline and the $g = 4.1$ signals is not accompanied by the decrease of the S_1-state signal in the presence of 3% methanol or 30% ethylene glycol. This may suggest that the S_1-state signal is not related to the Joliot−Kok S_1 state (Joliot et al., 1989; Kok et al., 1970). Actually, there is a clear linear relation between the decrease of the S_1-state signal intensity and the formation of the multiline signal in the absence of 3% methanol or in the presence of 50% glycerol (Dexheimer & Klein, 1994; this study). In addition, the spin quantification of the S_1-state signal showed that a majority of PS II centers (75 ± 25%) is responsible for this signal.

We, therefore, propose that some conformational alteration of the Mn cluster by these small alcohols leads to a modification of the exchange interaction responsible for the S_1-state signal. A change in the magnetic exchange interaction in the multiline Mn center on the addition of 3% methanol, from −3 to −13 K, is indicated (Pace et al., 1991). Furthermore, an electron nuclear double resonance (ENDOR) study of the multiline signal has led to the conclusion that ethylene glycol, unlike the larger glycerol molecule, can move close to the Mn cluster (Kawamori et al., 1989). It is therefore possible that the observed differences in the effects of alcohols on the S_1-state signal reflect their ability to access the Mn cluster, which is governed by their molecular size. It seems reasonable to assume an alcohol-induced modification of the Mn cluster occurs even in the S_1 state, although the above-mentioned results provide evidence for a modification in the S_2 state. We, therefore, expect that the modification of the Mn cluster caused by low-molecular weight alcohols (methanol or ethylene glycol) induces an EPR-silent S_1 state.

The temperature dependence of the S_1-state signal intensity has shown that the signal can be assigned to an excited state (with $S = 1$), lying 2.5 K above the ground singlet state (with $S = 0$). However, this result does not provide direct structural information regarding the origin of the S_1-state signal. The structure of the Mn cluster is still unresolved except for the presence of one or two dimers of Mn in which two manganese atoms are separated by 2.7 Å, probably through di-μ-oxo bridging (Yachandra et al., 1986). In principle, it is possible that a weakly exchange-coupled Mn(II)−Mn(II), Mn(III)−Mn(III), or Mn(IV)−Mn(IV) dimer generates the S_1-state signal. The stoichiometric relation between the decrease of the S_1-state signal and the formation of the multiline signal suggests that the Mn center responsible for the S_1-state signal generates the multiline signal with an exchange coupling of approximately -2 cm^{-1} after one-electron oxidation. Taking into account the involvement of an antiferromagnetically coupled Mn(III)−Mn(IV) dimer in the formation of the S_2-multiline signal, it is reasonable to suggest that a Mn(III)−Mn(III) dimer is responsible for generating the S_1-state signal. The absolute value of the exchange coupling constant of 0.87 cm^{-1} for the S_1-state Mn cluster in the absence of low-molecular weight alcohols will be more than 5 cm^{-1} in the presence of alcohols, resulting in the loss of the S_1-state signal due to a decrease in the Boltzmann population in the excited states. Such small changes in the exchange coupling constant induced by alcohols should not influence significantly the molecular structure of the S_2 state responsible to the multiline signal. In fact, no detectable alterations occurred in the hyperfine structure of the multiline signal. Alternatively, as demonstrated by a model compound consisting of a pair of di-μ-oxo-bridged Mn(III)−Mn(IV) dimers (Chan & Armstrong, 1991), the Mn cluster based on a dimeric dimer model could generate the S_1-state signal through exchange coupling between the respective dimers. In this case, a low-molecular weight alcohol would change the magnetic interaction between the two dimers, resulting in the disappearance of the S_1-state signal. Since the S_1 state has manifested only a weak paramagnetic susceptibility (Babcock et al., 1990; Sivaraja et al., 1989), the effective magnetic moment in the S_1 state may have a rather small value similar to the value presented by an organic radical. Furthermore, it manifested no relaxation enhancement of the tyrosine D radical (Koulougliotis et al., 1992; Un et al., 1995). These experimental results have led several groups to suggest that the S_1 state is diamagnetic. The $S = 1$ spin state may be the only excited state to yield the small effective magnetic moment, if the exchange interaction within each dimer in the S_1 state is strong enough to conquer thermal energy. Therefore, the dimer model suggested by Chang and Armstrong (1991) seems to be most reasonable.

As noted by Dexheimer and Klein (1992), there is no plausible explanation for the absence of the decrease in the intensity of the S_1-state signal when the $g = 4.1$ signal is induced with 140 K illumination. A recent multifrequency EPR study suggests that the Mn cluster consists of two magnetically isolated Mn dimers. One dimer is responsible for a ground state $g = 4.1$ signal in the S_2 state produced upon 140 K illumination, while the other is responsible for the parallel polarization signal in the S_1 state and generates the multiline as a ground state with $S = {}^1/_2$ and $g = 4.1$ signal as the first excited state upon illumination at 200 K (Smith & Pace, 1996). This view seems to be consistent with our experimental results but is difficult to reconcile with the finding by Boussac et al. (1996) that conversion of the multiline to the $g = 4.1$ signal may be induced upon illumination by infrared light (860 nm) at about 150 K. The conversion was interpreted as an intervalence charge transfer within the di-μ-oxo Mn(III)−Mn(IV) dimer. Kusunoki (1992) has proposed a model of the Mn cluster which involves two thermally dependent conformations in a single tetranuclear Mn cluster in both the S_1 and S_2 states: S_1-state signal and EPR silent conformations in the S_1 state and $g = 4.1$ and multiline signal conformations in the S_2 state. One transition from the S_1 signal state to the S_2-multiline state is stabilized as shown by the linear relation in Figure 5. The transition from the EPR-silent S_1 state preferentially occurs to the $g = 4.1$ signal S_2 state, which is stabilized to the S_2-multiline state through valence swapping between Mn(III) and Mn(IV). This heterogeneous S-state model seems not only to explain the unresolved relation between the S_1 state and $g = 4.1$ signals in this work but also to be compatible with the results reported by Boussac et al. (1996). Further accurate quantification of the S_1 state and $g = 4.1$ signal intensities will prove the reality of the models.

In conclusion, the present result has proved that the S_1 state is an excited paramagnetic state with spin 1 occurring within an exchange-coupled polynuclear Mn cluster. The separation of this spin 1 state from the ground singlet state is 2.5 K, which corresponds to a weak antiferromagnetic coupling with a J value of -0.87 cm^{-1}.

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REFERENCES

Åhling, K. A., & Pace, R. J. (1995) *Biophys. J. 68*, 2081−2090.

Astashkin, A. V., Kodera, Y., & Kawamori, A. (1994) *J. Magn. Reson. 105*, 113−119.

Babcock, G. T., Barry, B. A., de Paula, J. C., Mohamed, E. D., Petersen, J., Debus, R. J., Sithole, I., McIntosh, L., Bowlby, N. R., Dekker, J., & Yocum, C. F. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. 1, pp 239−246, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
Boussac, A., Girerd, J., & Rutherford, A. W. (1996) *Biochemistry 35*, 6984−6989.
Britt, R. D., Lorigan, G., Sauer, K., Klein, M. P., & Zimmmermann, J.-L. (1992) *Biochim. Biophys. Acta 1040*, 95−101.
Brudvig, G. W. (1995) in *Mechanistic Bioinorganic Chemistry* (Thorp, H. H., & Pecoraro,V. L., Eds.) pp 250−263, American Chemical Society, Washington, DC.
Carrington, A., & McLachlan, A. D. (1967) in *Introduction to Magnetic Resonance*, Chapter 8, Harper & Row, New York.
Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21−28.
Chan, M. K., & Armstrong, W. H. (1991) *J. Am. Chem. Soc. 113*, 5055−5075.
Dau, H., Andrews, J. C., Roelofs, T. A., Latimer, M J., Liang, W., Yachandra, V. K., Sauer, K., & Klein, M. P. (1995) *Biochemistry 34*, 5274−5287.
de Paula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114−8120.
de Paula, J. C., Beck, W. F., Miller, A.-F., Wilson, R. B., & Brudvig, G. W. (1987) *J. Chem. Soc., Faraday Trans. 1*, *83*, 3635−3651.
Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
Dexheimer, S. L., & Klein, M. P. (1992) *J. Am. Chem. Soc. 114*, 2821−2826.
Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274−278.
Haddy, A., Dunham, W. R., Sands, R. H., & Aasa, R (1992) *Biochim. Biophys. Acta 1099*, 25−34.
Hansson, O., Aasa, R., & Vanngard, T. (1987) *Biophys. J. 51*, 825−832.
Joliot, P., Barbieri, G., & Chabaud, R. (1969) *Photochem. Photobiol. 10*, 309−329.
Kawamori, A., Inui, T., Ono, T.-A., & Inoue, Y. (1989) *FEBS Lett. 254*, 219−224.
Kodera, Y., Dzuba, S. A., Hara, H., & Kawamori, A. (1994) *Biochim. Biophys. Acta 1186*, 91−99.
Kodera, Y., Hara, H., Astashkin, A. V., Kawamori, A., & Ono, T.-A. (1995) *Biochim. Biophys. Acta 1232*, 43−51.
Kok, B., Forbusch, B., & McGloin, M. P. (1970) *Photochem. Photobiol. 11*, 457−475.
Koulougliotis, D., Hirsh, D. J., & Brudvig, G. W. (1992*) J. Am. Chem. Soc. 114*, 8322−8323.
Kusunoki, M. (1992) in *Research in Photosynthesis*, Vol. II, pp 297−300, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Kuwabara, T., & Murata, N. (1982) *Plant Cell Physiol. 23*, 533−539.
Liang, W., Latimer, M. J., Dau, H., Roelofs, T. A., Yachandra, V. K., Sauer, K., & Klein, M. P. (1994) *Biochemistry 33*, 4923−4932.
Ono, T., & Inoue, Y. (1986) *Biochim. Biophys. Acta 850*, 380−389.
Ono, T., & Inoue, Y. (1992) *Biochemistry 31*, 5953−5956.
Ono, T., Zimmermann, J.-L., Inoue, Y., & Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 193−201.
Ono, T., Noguchi, T., Inoue, Y., Kusunoki, M., Matsushita, T., & Oyanagi, H. (1992) *Science 258*, 1335−1337.
Pace, R. J., Smith, P., Bramley, R., & Stehlik, D. (1991) *Biochim. Biophys. Acta 1058*, 161−170.
Randall, D. W., Sturgeon, B. E., Ball, J. A., Lorigan, G. A., Chan, M. K., Klein, M. P., Armstrong, W. H., & Britt, R. D. (1995) *J. Am. Chem. Soc. 117*, 11780−11789.
Roelofs, T. A., Liang, W., Latimer, M. J., Cinco, R. M., Rompel, A., Andrews, J. C., Sauer, K., Yachandra, V. K., & Klein, M. P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 3335−3340.
Rutherford, A. W., Zimmermann, J.-L., & Boussac, A. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 179−229, Elsevier, Amsterdam, The Netherlands.
Sivaraja, M., Philo, J. S., Lary, J., & Dismukes, G. C. (1989) *J. Am. Chem. Soc. 111*, 3221−3225.
Smith, P. J., & Pace, R. J. (1996) *Biochim. Biophys. Acta 1275*, 213−220.
Smith, P. J., Åhling, K. A., & Pace, R. J. (1993) *J. Chem. Soc., Faraday Trans. I 89*, 2863−2868.
Styring, S., & Rutherford, A. W. (1988) *Biochim. Biophys. Acta 933*, 378−387.
Un, S., Atta, M., Fontecave, M., & Rutherford, A. W. (1995) *J. Am. Chem. Soc. 117*, 10713−11719.
Yachandra, V. K., Guiles, R. D., MacDermott, A., Britt, R. D., Dexheimer, S. L., Sauer, K., & Klein, M. P. (1986) *Biochim. Biophys. Acta 850*, 324−332.
Zimmermann, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160−167.
Zimmermann, J.-L., & Rutherford, A. W. (1986) *Biochemistry 25*, 4609−4615.

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