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# SQUID Magnetization Study of the Infrared-Induced Spin Transition in the $S_2$ State of Photosystem II: Spin Value Associated with the $g = 4.1$ EPR Signal

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**Abstract:** The $Mn_4$ complex which is involved in water oxidation in photosystem II is known to exhibit three types of EPR signals in the $S_2$ state, one of the five redox states of the enzyme cycle: a multiline signal (spin $^1/_2$), signals at $g > 5$ (spin $^5/_2$), and a signal at $g = 4.1$ (spin value $^3/_2$ or $^5/_2$). The multiline and $g = 4.1$ signals are those the most readily observed. The relative proportions of the $g = 4.1$ signal and of the multiline signal are affected by many biochemical treatments including the substitution of $Ca^{2+}$and $Cl^-$ which are two essential cofactors for $O_2$ evolution. The state responsible for the multiline signal can also be converted, reversibly, to that responsible for the $g = 4.1$ signal upon the absorption of near-IR light at around 150 K. These infrared-induced effects are confined to the $Mn_4$ cluster, and no other redox change occurs in the enzyme. Here, we have used the IR-induced photochemistry of the $Mn_4$ cluster to measure the changes in magnetization occurring upon interconversion of the state responsible for the spin $^1/_2$ state and the $g = 4.1$ state. Measurements were performed with a SQUID magnetometer below 20 K and at magnetic fields $\leq$5.5 T. Simulations of experimental data provide strong indication that the spin value of the state responsible for the $g = 4.1$ state is $^5/_2$. Results are discussed in terms of a model implying an IR-triggered spin conversion of the $Mn^{III}$ (from the spin 2 to spin 1) of the $Mn_4$ cluster.

## Introduction

The evolution of oxygen as a result of light-driven water oxidation is catalyzed by photosystem II (PSII) in which a cluster of four manganese ions acts both as a charge accumulating device and as the active site.[1] The $Mn_4$ is known to exhibit in the $S_2$ state, one of the five redox states[1,2] of the enzyme cycle, a multiline EPR signal[1,3] (spin $^1/_2$), signals at $g > 5$[4] (spin $^5/_2$), and a signal at $g = 4.1$[5] (or $g = 4.25$ depending on experimental or preparation conditions). The multiline and $g = 4.1$ signals are those the most readily observed, and their relative proportion is affected by many biochemical treatments including the substitution of $Ca^{2+}$[1,6] and $Cl^-$[1,5a,7] which are two essential cofactors for $O_2$ evolution.[1] Knowledge of the spin value of the $Mn_4$ cluster in the state responsible for the $g \approx 4$ EPR signal is important for understanding the mechanism of the multiline to $g \approx 4$ conversion. Furthermore, this may give information on the structural and mechanistic roles of $Ca^{2+}$ and $Cl^-$. At present, the spin value of the $g = 4.1$ signal is under debate and is proposed to be either $^3/_2$[8] or $^5/_2$.[9,10]

The spin state of paramagnets can in principle be determined by measurements of magnetization.[11] This method has had only limited applications in the study of the PSII $Mn_4$ cluster[12] because the enzyme contains several other paramagnets and light-induced difference studies are hindered by magnetic changes associated with the electron acceptors.[1] Recently, it was shown that, in $S_2$, the state responsible for the multiline EPR signal at 10 K (called here the spin $^1/_2$ state) can be

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**Table 1.** Results of the Simulation Procedure for Spin 5/2[a]

| | | | *E*/*D* | | | | | |
|---|---|---|---|---|---|---|---|---|
| experiment | $Mn_4$-cluster (nmole) | | **0.25** | **0.29** | **0.33** | **0.25** | **0.29** | **0.33** |
| isotherm (Figure 3) | ≤11.0 | D ($cm^{-1}$) | 1.7[b] | 1.4[c] | 1.2[d] | −1.05[e] | −1.10[f] | −1.20[g] |
| | | N (nmole) | 3.45[b] | 3.39[c] | 3.34[d] | 3.28[e] | 3.30[f] | 3.34[g] |
| isofield (Figure 4) | ≤9.5 | D ($cm^{-1}$) | **1.7** | **1.4** | **1.20** | **−1.05** | **−1.10** | **−1.20** |
| | | N (nmole) | 2.54 | 2.50 | 2.48 | 2.44 | 2.46 | 2.47 |

[a] The mole number of $Mn_4$-clusters in the two different PSII samples which have been used was estimated by atomic absorption as indicated in the text. The *N* value represents the mole number of $Mn_4$-clusters which were affected by the near-infrared illumination. The values in bold characters are those fixed in the simulation procedure. [b] Panel A. [c] Panel B. [d] Panel C. [e] Panel D. [f] Panel E. [g] Panel F.

reversibly converted to the state responsible for the $g = 4.1$ EPR signal at 10 K (called here the $g = 4.1$ state) through the absorption of IR light.[4,13] Since no other redox reactions take place under these conditions, we have used this IR-induced photochemistry to study the changes in magnetization associated with the spin $^1/_2$ state to $g = 4.1$ state conversion. It is shown that the $g = 4.1$ state arises from a spin $^5/_2$ state.

## Materials and Methods

The $O_2$-evolving PSII samples were prepared as previously described[4a,13] except that two additional washings in $D_2O$ buffer containing 50 $\mu$M EDTA, 10 mM NaCl, 0.3 M sucrose, and 25 mM Mes pD 6.5 were performed. The membranes (taken directly as the pellet of the last centrifugation) were loaded into a symmetrical sample holder. This sample holder consisted of two 5 mm (outer diameter) quartz EPR tube which were fused together end-to-end. One of which was previously sealed empty under high vacuum ($\sim 2\ 10^{-5}$ mbar). After dark-adaptation for 1 h at 0 °C, 1 mM phenyl-*p*-benzoquinone dissolved in ethanol[4a] (95%) was added to the sample as an electron acceptor. Formation of the $S_2$ state was achieved by illumination of the samples with a 800 W tungsten lamp filtered through water (which absorbs above 900 nm) and IR filters (cut off above 750 nm) in a non-silvered dewar at 200 K (ethanol, solid $CO_2$). The sample was then degassed at 200 K under vacuum ($\sim 2\ 10^{-5}$ mbar)[4] and transferred to 77 K before being sealed. Near-IR illumination of the samples, at 150 K, was done in a nitrogen gas flow system (Bruker, B−VT-1000). IR illumination was provided by a laser diode emitting at 813 nm (Coherent, diode S-81−1000C) with a power of 600−700 mW at the level of the sample.[4]

CW-EPR spectra were recorded at liquid helium temperatures as previously described[4,13] with a Bruker ESP300 X-band spectrometer equipped with an Oxford Instruments cryostat. Magnetization measurements were performed with a MPMS5 SQUID magnetometer (Quantum Design Inc.). Mn content of the PSII samples used in the magnetization experiments was determined by atomic absorption spectrometry with a Perkin-Elmer 2280 graphite furnace. The Mn standard was purchased from Aldrich. Given that there are 4 Mn ions per PSII,[1] an upper value of the amount of $O_2$-evolving PSII was estimated by dividing the total Mn content by 4 (Table 1).

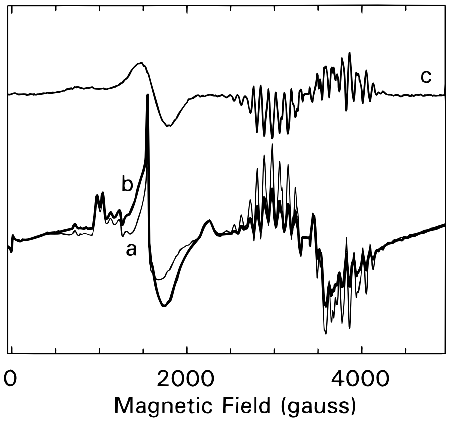

**Figure 1.** EPR spectra of oxygen-evolving PSII recorded at 10 K after a 200 K illumination (spectrum a) and a further 813 nm illumination given at 150 K (spectrum b). Spectrum c was obtained by subtracting the spectrum recorded before the IR illumination from that recorded after the IR illumination. Instrument settings: temperature, 10 K; modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz. The central part of the spectra corresponding to the $TyrD^\bullet$ region was deleted.

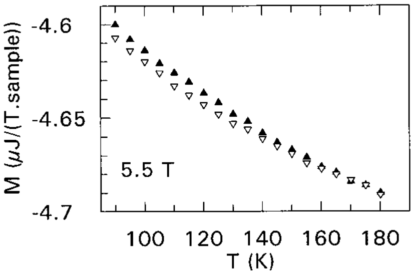

**Figure 2.** Sample magnetization at 5.5 T measured after a 200 K illumination and a further 813 nm illumination given at 150 K. The measurements were made from 100 to 180 K (▲) then from 180 to 100 K (▽).

## Results and Discussion

In dark-adapted $O_2$-evolving PSII, the proportion of reaction centers which are in the $S_1$ $Tyr_D^\bullet$ state is ≥75% (the other centers are in the $S_0$ $Tyr_D^\bullet$ state or in the $S_1$ $Tyr_D$ state[14]). Formation of the $S_2$ state may be achieved by illumination at 200 K of PSII membranes in the $S_1$ state. As an EPR control, the extent of the multiline signal in the $S_2$ state was measured in the same PSII preparations as those used in the magnetization measurements (spectrum a, Figure 1).

Spectrum b in Figure 1 was recorded after a further near-IR illumination at 150 K. Spectrum c is the difference spectrum: spectrum b *minus* spectrum a. The signal which disappeared upon IR illumination (i.e., a fraction of the multiline signal) appears negative and the IR-induced signal (i.e., the $g = 4.1$ signal at ~1600 G) appears positive. From the proportion of the multiline signal which disappeared under IR illumination, it can be deduced that IR light converted the spin $^1/_2$ state into the $g = 4.1$ state in ~50% of the PSII membranes used in the present work.[13b]

For magnetization measurements, the dark-adapted PSII samples were first illuminated at 200 K to form the $S_2$ state and subsequently the $g = 4.1$ state was generated by an IR

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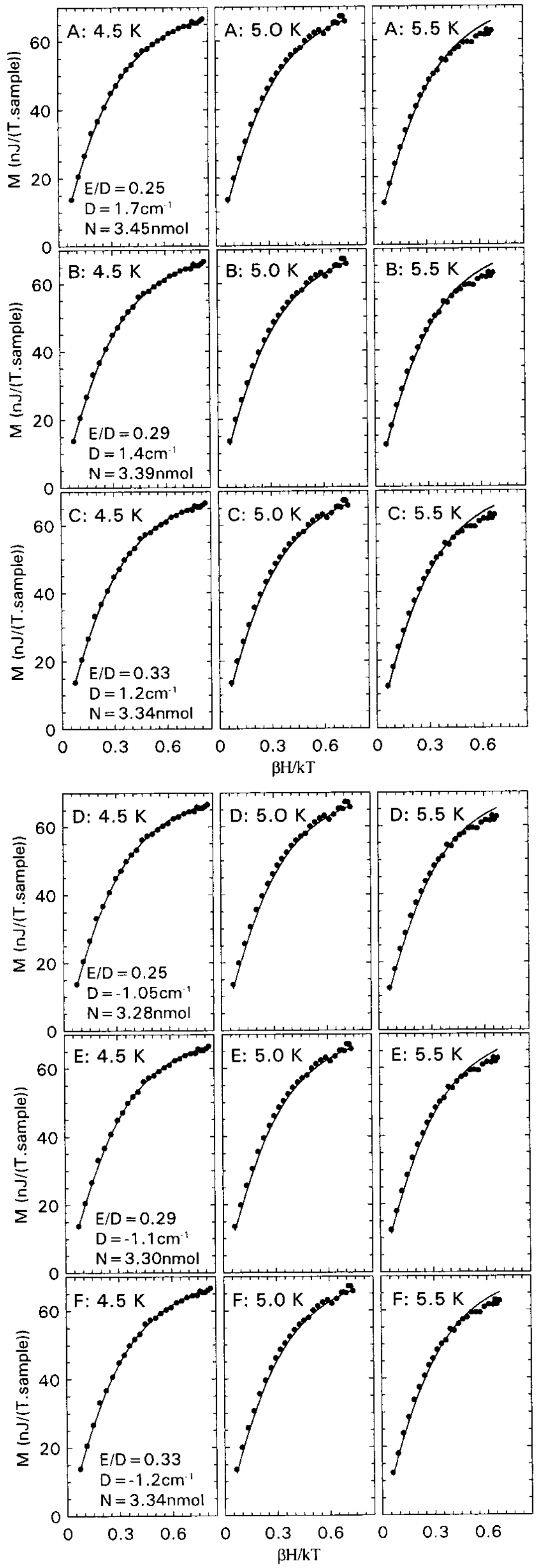

**Figure 3.** Isotherm experiments performed at 4.5, 5.0, and 5.5 K. The sample magnetization shown corresponds to the magnetization measured after a 200 K illumination followed by a 813 nm illumination given at 150 K *minus* that measured after warming of the sample at 180 K in the magnetometer. The lines through the points correspond to simulations for $S = {}^{5}/_{2}$ with an $E/D$ ratio fixed to 0.25 (panels A and D), with $E/D = 0.29$ (panels B and E), and with $E/D = 0.33$ (panels C and F). Panels A−C, show the best fits obtained with a positive $D$ value, and panels D−F show the best fits obtained with a negative $D$ value. Simulation were carried out simultaneously on data obtained at the three temperatures.

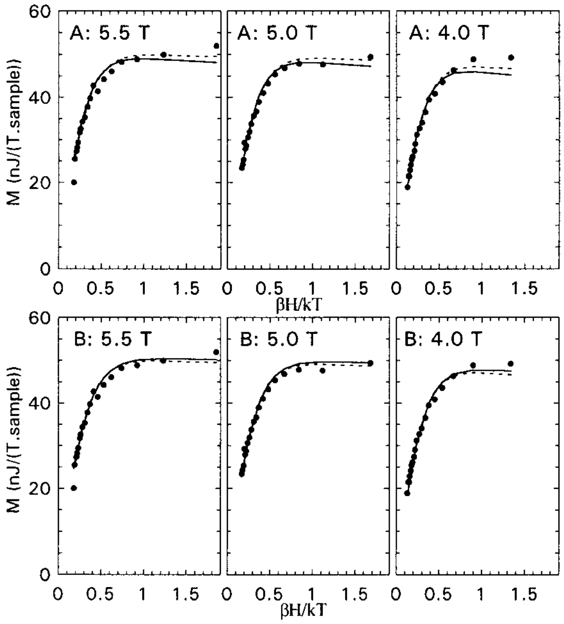

**Figure 4.** Isofield experiments performed at 5.5, 5.0, and 4.0 T. The lines through the points correspond to simulations for $S = {}^{5}/_{2}$ with a $E/D$ ratio fixed either to 0.25 (continuous lines) or to 0.33 (dashed lines). The $D$ values (positive in panels A and negative in panels B) which have been used are indicated in Table 1.

illumination at 150 K. After undergoing these illumination procedures, the samples were transferred immediately to liquid nitrogen and then transferred at 50 K to the magnetometer. After an incubation at 100 K allowing $O_2$ condensed on the outside of the tube to be removed by evaporation,[11] magnetization of the samples was measured at various magnetic fields and temperatures.

Closed triangles in Figure 2 show the magnetization, at 5.5 T, of a PSII sample after the IR illumination, i.e., in the $g = 4.1$ state. In this experiment, the temperature was increased from 100 to 180 K. Increasing the temperature in this range allows the IR-triggered spin conversion to revert progressively[4,13] into the spin ${}^{1}/_{2}$ state. The spin value of the $g = 4.1$ state is expected to be higher than that responsible for the spin ${}^{1}/_{2}$ state. Indeed, the new set of magnetization measurements performed by decreasing the temperature from 180 to 100 K (Figure 2, open triangles) revealed a decrease in the magnetization of the sample. The difference between the two curves, below 150 K, is proportional to the amount of the reaction centers which were affected by IR light and corresponds to the difference in the magnetization of the $g = 4.1$ state and that of the spin ${}^{1}/_{2}$ state. This difference in the magnetization of the two forms of the $S_2$ state will be shown below versus $\beta H/kT$ at a constant temperature (isotherm curves) or versus $\beta H/kT$ at a constant magnetic field (isofield curves).[11] Figures 3−5 show data recorded below 20 K to avoid possible complications due to the population of excited states.

Simulations of the isotherm and isofield curves were performed with a FORTRAN program assuming either a spin ${}^{5}/_{2}$ or a spin ${}^{3}/_{2}$ for the $g = 4.1$ state. First, the magnetization value for a spin state ($S = {}^{5}/_{2}$ or ${}^{3}/_{2}$) was calculated according to the relation $M_i = Ng_e\beta\langle S\rangle_{Ti}$, where $\langle S\rangle_{Ti}$ corresponds to the $i$th component of the thermally averaged spin value and $N$ corresponded to the number of moles of IR-susceptible PSII. The $g$ value was fixed isotropic at 2.0. For the $g = 4.1$ state, the classical spin Hamiltonian $H = D\{[S_z^2 - S(S + 1)/3] + (E/D)(S_x^2 - S_y^2)\} + \beta \mathbf{S}\cdot\mathbf{g}\cdot\mathbf{H}$ was used. Second, the difference between the magnetization value of the $g = 4.1$ state and that,

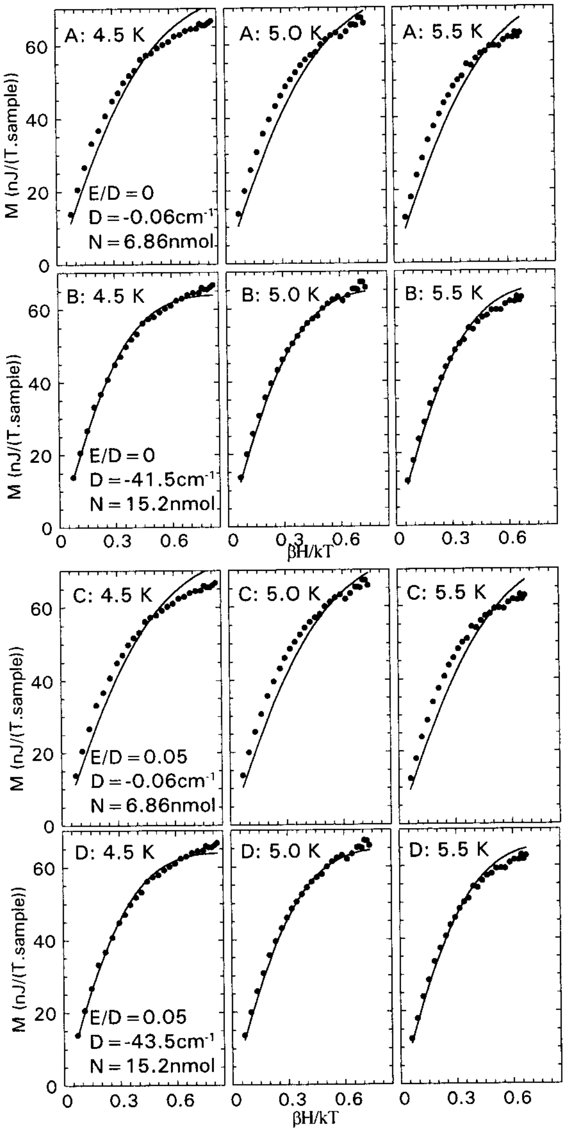

**Figure 5.** Isotherm experiments performed at 4.5, 5.0, and 5.5 K (same data as in Figure 3). The lines through the points correspond to the best simulations for $S = {}^3/_2$ obtained with a $E/D$ ratio either fixed to 0 (panels A and B) or fixed to 0.05 (panels C and D).

multiplied by $N$, associated with a spin $^1/_2$ state (i.e., a Brillouin law) was then calculated.

For a spin $^5/_2$, the $E/D$ ratio was varied between 0.25 and 0.33 to account for the rhombicity. For a spin $^3/_2$, the $E/D$ ratio was varied between 0.05 and 0.0 to account for the axiality. The value of the zero-field splitting parameters $D$ and that of the number of moles of IR-susceptible PSII ($N$) were obtained from the simulation of the isotherm experiments. The isofield curves were simulated by using the $D$ values obtained from the isotherm fittings, and therefore, $N$ was the only variable in this case.

Figure 3 shows isotherm experiments performed at 4.5, 5.0 and 5.5 K. Figure 3 also shows example of simulation curves (lines through the points) for $S = {}^5/_2$ with an $E/D$ ratio fixed to 0.25 (panels A and D), with $E/D = 0.29$ (panels B and E), and with $E/D = 0.33$ (panels C and F). Panels A−C show the best fits obtained with a positive $D$ value and panels D−F show the best fits obtained with a negative $D$ value. Simulation were carried out simultaneously on data obtained at the 3 temperatures. The results of the simulations are summarized in Table 1.

Figure 4 shows isofield experiments performed at 5.5, 5.0, and 4.0 T with a different batch of PSII membranes. Figure 4 also shows example of simulation curves for $S = {}^5/_2$ with a $E/D$ ratio fixed either to 0.25 (continuous lines) or to 0.33 (dashed lines). The $D$ values (positive in panels A and negative in panels B) which have been used are indicated in Table 1. Simulation were carried out simultaneously on data obtained at the three magnetic fields. The $N$ values obtained from the simulations are presented in Table 1.

For spin $^5/_2$, the best simulations were obtained with $D$ values equal to 1.7 cm$^{-1}$ or $-1.05$ cm$^{-1}$ for $E/D = 0.25$ (in these experiments the sign of $D$ cannot be determined). Figures 3 and 4 and Table 1 show that varying the $E/D$ values from 0.25 to 0.33 had little influence on the $N$ value. The $N$ value obtained from the fit corresponded to an IR-triggered spin conversion in ~30% of the PSII centers. This estimate of N seems reasonable considering (i) the fact that the estimate of the mole number of PSII centers is an upper value, (ii) the yield of the spin $^1/_2$ to $g = 4.1$ conversion (~50% in the matching EPR sample), and (iii) given the difficulties in mounting the illuminated sample in the magnetometer, the yield of conversion estimated from the EPR must be considered as an upper limit when applied to magnetization experiments.

For spin $^3/_2$, we were unable to obtain parameters giving a satisfactory fit. The best simulations either poorly fitted the experimental points (e.g., panels A and C in Figure 5) or resulted in $D$ values incompatible with the observation of an EPR signal at $g = 4.1$ and/or in $N$ values greater than the mole number of PSII center in the sample (e.g., panels B and D in Figure 5).

Earlier magnetization measurements performed on PSII, at room temperature, were able to detect an increase in the magnetization during the advancement of the S state cycle but were unable to detect a difference in magnetization between the $g = 4.1$ state and the spin $^1/_2$ state.[12] Here, we show that using the IR-triggered spin $^1/_2$ to $g = 4.1$ state conversion, it is possible to detect the associated changes in the magnetization of the $Mn_4$ cluster. The two spin values (i.e. $S = {}^5/_2$[9,10] or $S = {}^3/_2$[8]) for the $g = 4.1$ state have been proposed in the literature.[15] Results from SQUID magnetization experiments strongly indicate that the spin value of the $g = 4.1$ state is $^5/_2$.

Recently, it was shown that IR illumination at temperatures lower that 77 K converted the spin $^1/_2$ state to a third form of the $S_2$ state which has been attributed to a spin state $\geq {}^5/_2$[4] and which is characterized by EPR signals at $g = 10$ and $g = 6$ in PSII from spinach.[4a] Upon warming of the sample above 77 K, the signals at $g = 10$ and $g = 6$ converted to that at $g = 4.1$.[13] The changes in the zero-field splitting parameters occurring upon warming and associated with this transition may reflect a relaxation in the ligand environment: that giving rise to the $g = 10$ and $g = 6$ signals (with $E/D = -0.05$ and $|D| \leq 1$ cm$^{-1}$) being electronically more symmetrical.

The most commonly favored structure for the $Mn_4$ cluster in the $S_2$ state includes a di-$\mu$-oxo($Mn^{III}Mn^{IV}$) motif.[1,3] The IR effect has been explained either by an electron transfer from the $Mn^{III}$ to the $Mn^{IV}$ or by a spin state transition in the $Mn^{III}$ ion from spin 2 (high spin) to spin 1 (low spin).[4,13] This latter model is based on the light-induced electron spin transition effect observed in iron photochemistry.[17] In this model, the spin 5/2

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(15) The $g = 4.1$ EPR signal has been proposed to arise either from an excited state (spin $^3/_2$) or from a ground state (spin $^5/_2$ or $^3/_2$) depending on the experimental conditions in which the $g = 4.1$ EPR signal was induced.[16] The $g = 4.1$ signal proposed to arise from a spin $^5/_2$ ground state in ref 10 has been formed in conditions which are proposed to favor the formation of the spin $^3/_2$ excited state in ref 16. The IR-induced $g = 4.1$ signal (demonstrated here to arise from a spin $^5/_2$ ground state) corresponds to the $g = 4.1$ signal in ref 5a which is attributed to a ground state in ref 16.

value would originate from a double exchange phenomenon (as proposed in iron complexes[18]) between the low spin $Mn^{III}$ ion and the $Mn^{IV}$ ion. In such a model, the biochemical treatments which influence the yield of the $g = 4.1$ state would be seen as affecting the spin state of the $Mn^{III}$ ion while in the $Mn^{III}$ to $Mn^{IV}$ charge-transfer model the biochemical treatments may be seen as influencing the electrostatic environment of the $Mn_4$ cluster.

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