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Comparative study of the $g = 4.1$ EPR signals in the S_2 state of photosystem II

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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 $g = 4.1$ (or $g = 4.25$). The $g = 4.1$ signal could be generated under two distinct sets of conditions: either by illumination at room temperature or at 200 K in certain experimental conditions ($g4^S$ signal) or by near-infrared illumination between ≈ 77 and ≈ 160 K of the S_2-multiline state ($g4^{IR}$ signal). The two $g = 4.1$ signals arise from states which have quite different stability in terms of temperature. In the present work we have compared these two signals in order to test if they originate from the same or from different chemical origins. The microwave power saturation properties of the two signals measured at 4.2 K were found to be virtually identical. Their temperature dependencies measured at non-saturating powers were also identical. The presence of Curie law behavior for the $g4^S$ and $g4^{IR}$ signals indicates that the states responsible for both signals are ground states. The orientation dependence, anisotropy and resolved hyperfine structure of the two $g4$ signals were also found to be virtually indistinguishable. We have been unable to confirm the behavior reported earlier indicating that the $g4^S$ signal is an excited state, nor were we able to confirm the presence of signal from a higher excited state in samples containing the $g4^S$, nor a radical signal in samples containing the $g4^{IR}$. These findings are best interpreted assuming that the two signals have a common origin i.e. a spin 5/2 ground state arising from a magnetically coupled Mn-cluster of 4 Mn ions.

Keywords: Near-infrared; Oxygen evolution; Mn cluster; Spin state transition

1. 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 oxidizing accumulating device and as the active site. In the S_2 state, one of the five redox states of the enzyme cycle [1–6], the Mn_4-cluster exhibits a multiline EPR signal (spin 1/2) [2–9], signals at $g > 5$ [10,11] (spin 5/2) and a signal at $g = 4.1$ (or $g = 4.25$ depending on experimental conditions). The $g = 4.1$ signal could be generated under two distinct sets of

Abbreviations: P_{680}, photooxidizable chlorophyll (Chl) of photosystem II (PSII); Tyr_Z, the tyrosine acting as the electron donor to P_{680}; Tyr_D, the tyrosine acting as a side path electron donor to P_{680}; Q_A, primary quinone electron acceptor of PSII; EPR, electron paramagnetic resonance; PPBQ, phenyl-*p*-benzoquinone; MES, 2-(*N*-morpholino) ethanesulfonic acid; EDTA, ethylene diamine tetra acetic acid; IR, infrared; DMSO, dimethyl sulfoxide

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conditions: either by illumination at room temperature or at 200 K in the presence of sucrose [12,13] or by illumination at 140 K [14,15]. In the latter case, the signal generated at 140 K was lost upon warming to 200 K with the simultaneous appearance of the S_2-multiline signal [14]. Thus, the two conditions for generating the $g=4.1$ signal produced states which had a quite different stability in terms of temperature.

The generation of the $g=4.1$ signal at 140 K has recently being shown to be the result of two photochemical events: the first being photosynthetic charge separation resulting in an S_2 state which gives rise to the multiline signal, the second being the infrared-induced conversion of this state to the $g=4.1$ state due to the presence of 820 nm light in the broad-band illumination given [16]. In the following, this $g=4.1$ signal will be called $g4^{IR}$ signal.

For the $g=4.1$ signal that is stable at 200 K and above, the fraction of centers giving rise to this signal is dependent on the pretreatment of the enzyme, being markedly increased by: (1) having sucrose present in the medium [12,13]; (2) certain treatments which remove chloride from the medium [17,18] or its replacement by F^- [14,19], I^- [19,20], amines [21] or NO_3^- [19]; or (3) replacing Ca^{2+} with Sr^{2+} [22]. This $g=4.1$ signal is suppressed by the presence of alcohols [13]. In the following, this $g=4.1$ signal will be called $g4^S$ signal ('S' for stable).

The $g=4.1$ signal was proposed to arise from either a spin 3/2 state or spin 5/2 state [23–33] (see Table 1). The capacity of near-infrared light to induce a signal at $g=4.1$ allowed several observations in the literature on the $g4$ states to be rationalized [10,11,16,33]. Nevertheless, the question remains whether the $g4^{IR}$ signal and the $g4^S$ signal have different chemical origins and therefore if the properties found for the $g4^{IR}$ state can be extended to the $g4^S$ state. In the literature, the majority of studies have implicitly assumed that the $g4$ signals formed under different conditions arose from the same species (but see Table 1). However, it has been suggested recently that the two types of $g4$ signal arise from different Mn dimers, one of which, the $g4^S$ state, gives rise to an excited state, and the other, the $g4^{IR}$ state, gives rise to a ground state [24–27]. Furthermore, in order to obtain a high-spin ground state from an Mn dimer, an interaction with a third spin was postulated and evidence for coupling to a radical has been reported [24–27]. This situation is markedly different from the more generally held view that the Mn-cluster in the S_2 state consists of a magnetically coupled tetramer [2–9]. Therefore, the interpretation of the EPR spectroscopy has important repercussions on the current view of the structure of the Mn-cluster. In this work, the different $g4$ states have been generated and their properties were compared. This was done by using controlled conditions to generate specifically the $g4^{IR}$ and $g4^S$ signals.

2. Materials and methods

The O_2 evolving PSII samples were prepared as previously described [16] except that one additional washing in a buffer containing 50 μM EDTA, 10 mM NaCl, 25 mM MES pH 6.5 and either 0.3 or 0.5 M sucrose was performed. EDTA has been added to remove adventitious Mn^{2+} ions. The membranes (at ≈8–10 mg Chl/ml) were then loaded into a quartz EPR tube and dark-adapted for 1 h at 0°C. Then, 1 mM phenyl-*p*-benzoquinone dissolved either in ethanol (95%, Carlo Erba) or in DMSO was added to the samples containing 0.3 or 0.5 M sucrose, respectively. After dark-adaptation, the samples were degassed at 200 K under vacuum (≈5 10^{-2} mbar) and placed under a He atmosphere then transferred to liquid nitrogen (77 K). 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). NaCl-EGTA treatment and polypeptide-reconstitution of PSII were done as reported previously [34]. Orientation of these membranes on mylar sheets was done as previously described [35].

Near-IR illumination of the samples was done in a nitrogen gas flow system (Bruker, B-VT-1000 or B-VT-3000). 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.

CW-EPR spectra were recorded at liquid helium temperatures with a Bruker ESP300 X-band spectrometer equipped with an Oxford Instruments cryostat. The temperature at the sample level in the cryo-

stat was measured with a Rhodium-Iron temperature probe (Oxford Instruments).

3. Results

The nature of the states (i.e. ground state or excited state) from which the $g4^S$ and the $g4^{IR}$ signals originate can be determined by measuring the temperature dependence of the amplitude of the signals. In the present work, the temperature was varied between 4.2 and 30 K. For this experiment, a non-saturating microwave power must be used for recording of the spectra. Therefore, the highest non-saturating microwave power which could be used at the lowest temperature (i.e. 4.2 K) was determined. For that, the microwave power was varied between 200 mW and 0.25 μW at 4.2 K. Figs. 1 and 2 show the spectra recorded in two limiting conditions of microwave power: i.e. 4.2 K and 50 mW (Figs. 1A and 2A), 4.2 K and 25 μW (Figs. 1B and 2B). Figs. 1C and 2C show spectra recorded at 30 K with a microwave power of 100 μW which is the highest non-saturating microwave power at 4.2 K (see below).

In Fig. 1, the spectra labeled a, in each of the panels, were recorded in the dark-adapted state, i.e. in the S_1 state, and spectra b were recorded after illumination at 200 K, i.e. in the S_2 state. Spectra c correspond to the light-*minus*-dark spectra (i.e. spectra b *minus* spectra a). The illumination resulted in the formation of the S_2-multiline signal in about 55% of the centers (as estimated by comparison with the amplitude of the multiline signal formed in a similar sample with ethanol present) and in the formation of the $g4^S$ signal in about 45% of the centers. Under the conditions used, signals from the electron acceptor side are also visible in Fig. 1A: (1) the $Q_A^-Fe^{2+}$ signals at $g=1.9$ and 1.82; and (2) negative signals at $g=8$ and $g=5.7$ which are due to the light-induced reduction of Q_AFe^{3+} into Q_AFe^{2+} [36]. The presence of Q_AFe^{3+} in a small proportion of dark-adapted centers is indicative that Q_A^- (or Q_B^-) remained in these centers prior to the addition of PPBQ. The vertical scales in Fig. 1 allow the relative amplitudes of the signals measured in the three conditions to be compared.

In Fig. 2, spectra a were recorded after 200 K illumination, i.e. in the S_2 state, in a sample containing 0.3 M sucrose and 3% ethanol. Only a very small signal at $g=4.1$ was formed by the 200 K illumination under these conditions (less than 10% of that in Fig. 1, not shown). Spectra b were recorded after a further near-infrared illumination at 160 K. Spectra c are the differences, spectra b *minus* spectra a. IR illumination resulted in the appearance of the $g4^{IR}$ signal with a parallel loss of the multiline signal. Illumination at 160 K is not optimum for generating the $g4^{IR}$ signal with the highest yield. The highest

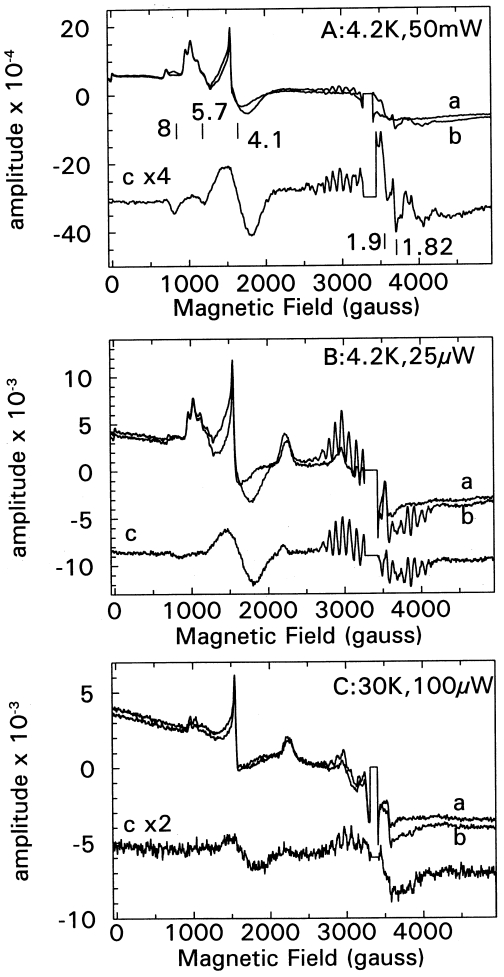

Fig. 1. EPR spectra recorded in dark-adapted PSII (spectra a) and after a 200-K illumination (spectra b). The temperature and the microwave power used for the recording of the spectra were 4.2 K and 50 mW in A, 4.2 K and 25 μW in B, and 30 K and 100 μW in C. Spectra c correspond to the light-*minus*-dark spectra. The amplitude of spectra c was multiplied by the indicated factors. Other instrument settings: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz. The central part of the spectra corresponding to the $Tyr_D^{\bullet}$ region was deleted.

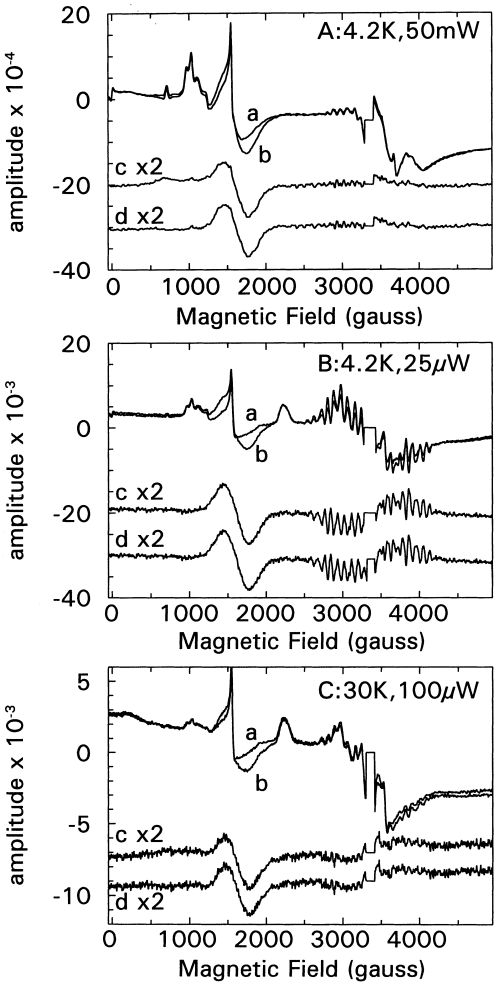

Fig. 2. EPR spectra recorded after a 200-K illumination (spectra a) and after a further IR illumination given at 160 K. The temperature and the microwave power used for the recording of the spectra were 4.2 K and 50 mW in A, 4.2 K and 25 μW in B, and 30 K and 100 μW in C. Spectra c correspond to the IR-induced spectra. Spectra d were obtained by subtracting the contribution of signals at $g>5$ present in spectra c. The amplitude of spectra c and d was multiplied by the indicated factors. Other instrument settings: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz. The central part of the spectra corresponding to the $Tyr_D^{\bullet}$ region was deleted.

yield is observed at 150 K, a temperature at which $\approx 60\%$ of the centers in the multiline state can be converted into the $g4^{IR}$ state. This yield decreased to $\approx 50\%$ at 160 K [10]. IR illumination at 160 K minimizes the proportion of centers in which signals at $g>5$ are trapped instead of the $g4^{IR}$ signal [10]. At 160 K, the $g>5$ signals are induced in less than 10% of the centers. Pure $g4^{IR}$ signals (spectra d) can be obtained by subtracting the contribution of the $g>5$ signals in spectra c. Signals at $g>5$ were obtained in separate experiments in which IR illumination was given below 77 K and in which spectra were measured with the same conditions as those described in Fig. 2. The vertical scales in Fig. 2 allow the relative amplitude of the signals measured in the three conditions to be compared. From the results in Figs. 1 and 2, it can be seen that the $g4^{IR}$ and $g4^{S}$ signals are well resolved in all the conditions used.

Figs. 1 and 2 also show the g_Z (at 2230 gauss) and the g_Y (at 2995 gauss) signals from the oxidized heme of cyt b_{559} and the S_2-multiline signal (between 2450 and 4300 gauss). These signals are saturated under the conditions used in Figs. 1A and 2A, but are clearly visible in Figs. 1B and 2B.

The microwave power dependence of the $g4^{IR}$ and $g4^{S}$ signals was determined by experiments shown in Fig. 3. The value, at 4.2 K, of the double integration between 700 and 2300 gauss of the $g4^{S}$ signal (Fig. 3A,B) and that of the $g4^{IR}$ signal (Fig. 3C,D) versus the square root of the microwave power has been plotted. Identical results were obtained by plotting the amplitude of the derivative signals instead of the area (not shown). The continuous lines through the data points in Fig. 3 correspond to the best fit using the equation $\text{Area} = k\sqrt{P}/(1+(P/P_{1/2}))^{b/2}$ [37], where b is the inhomogeneity parameter, P the microwave power, $P_{1/2}$ the microwave power at half saturation and k a factor which was adjusted to normalize the fitted curve to the experimental points. Both signals show a similar microwave power dependence and saturate with the same $P_{1/2}$ value, i.e. 1.5 mW at 4.2 K. Fig. 3B and D show the same data on an expanded x-scale. The dotted lines correspond to an extrapolation of the linear part of the fit. Fig. 3B and D show that at 4.2 K the highest non-saturating microwave power is 100 μW. Figs. 1C and 2C show that in these conditions both the $g4$ signals are well resolved in the samples used in these conditions.

The temperature dependence of the $g4^{IR}$ and $g4^{S}$ signals were examined by experiments shown in Fig. 4. The $g4$ signals were recorded with a microwave power of 100 μW and the amplitude of the double integrals were plotted versus the reciprocal of the temperature. The straight lines through the data points were obtained by linear regression. Both the

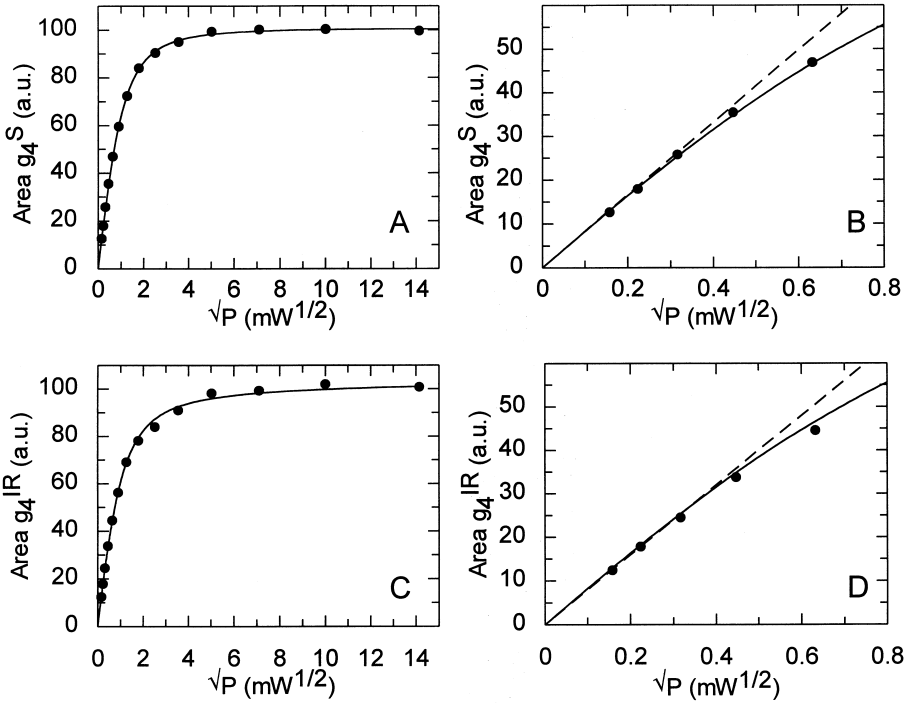

Fig. 3. Microwave power saturation of the $g4^S$ signal (A,B) and of the $g4^{IR}$ signal (C,D). The double integration of the spectra was plotted versus the square root of the microwave power. Spectra were recorded at 4.2 K in conditions described in Figs. 1 and 2. The continuous lines through the experimental points correspond to the best fit using the equation and the parameters described in text. The dashed lines in B and D correspond to an extrapolation of the linear part of the fitted curve below 100 μW.

$g4^{IR}$ signal and the $g4^S$ signal behave linearly with $1/T$. There is no indication of a deviation at the lowest temperature used. Moreover, at infinite temperature, the straight lines from the linear regression intercept the $1/T$ axis very close to its origin. Identical results were obtained by plotting the amplitude of the derivative signals (not shown). As a control, the temperature dependence of the non-heme iron signal at $g=8$ present in the sample (see Fig. 1A) was simulated by using a D value of 1 cm^{-1} which is in agreement with the value previously reported [38]. This confirms that the temperature values measured in Fig. 4 were correct.

The results presented above show that the S_2 state responsible for the $g4^{IR}$ and $g4^S$ signals follow Curie law behavior and therefore are ground states. Additionally, the two $g4$ states saturate at a similar microwave power.

Ca^{2+}-depleted, chelator-treated, polypeptides reconstituted PSII membranes exhibit a modified multiline signal [34] arising from an S_2 state which is stable in the dark. IR illumination of this sample resulted in the formation of a $g4^{IR}$ signal (in this case at $g=4.25$) [16]. Fig. 5 shows the result of IR illumination on this kind of material which has been oriented on mylar sheets. Fig. 5A shows the whole difference spectra (after IR illumination *minus* before IR illumination). When the normal of the membrane was parallel to the direction of the external magnetic field, the g value was 4.1 while when the normal of the membrane was perpendicular to the direction of the external magnetic field the g value was 4.3. The anisotropy of the $g4^{IR}$ signal in Ca^{2+}-depleted, chelator-treated, polypeptides reconstituted PSII membranes measured here is identical to that already in the literature of the $g4^S$ signal in untreated [35] and ammonia-treated [28,29] PSII membranes.

Fig. 5B and C show the $g=4$ region on an expanded magnetic field scale and spectra recorded with better resolution than in Fig. 5A. Spectra a were recorded before the IR illumination and spectra b were recorded after the IR illumination. Spectra c are the difference spectra. With the normal of the membranes parallel to the external magnetic field direction, a hyperfine structure is clearly visible. This structure is very similar to that previously de-

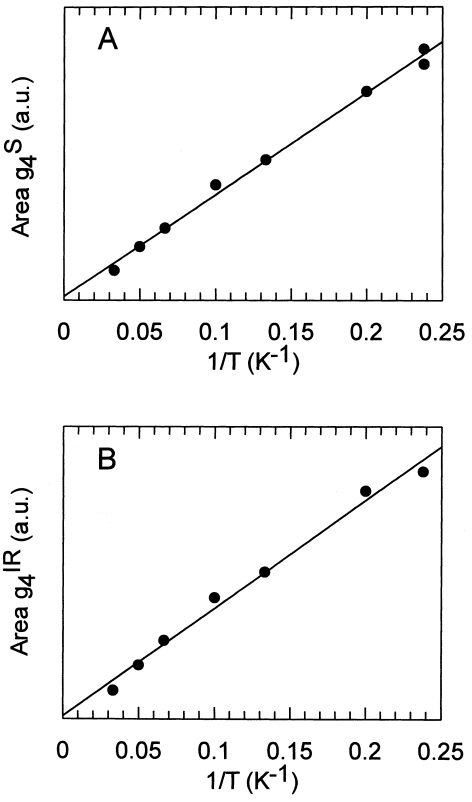

Fig. 4. Temperature dependence of the double integration of the $g4^S$ signal (A) and of the $g4^{IR}$ signal (B). Instrument settings: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 100 μW. The reproducibility of the temperature was better than ±0.2 K.

tected in the ammonia-treated sample, i.e. more than 16 lines spaced by 36 gauss [28,29]. Although less resolved, some hyperfine structure is also visible in the sample with the normal of the membrane oriented perpendicular to the magnetic field direction. Again, it is clear that the hyperfine structure found here for the $g4^{IR}$ signal in the Ca^{2+}-depleted, chelator-treated, polypeptide reconstituted PSII is similar to that reported earlier for the $g4^S$ signal in ammonia-treated PSII.

As mentioned in the introduction, it has been suggested that the two different $g4$ signals arise from different Mn dimers [24–27]. In this model, the $g4^S$ signal ($S=3/2$) is part of a spin system (i.e. a $Mn^{III}Mn^{IV}$ dimer) where a higher excited state ($S=5/2$) exists. This state has been reported as giving rise to an EPR signal at $g=6$ and detection of this state is favored at $\approx$30 K. This possibility was ad-

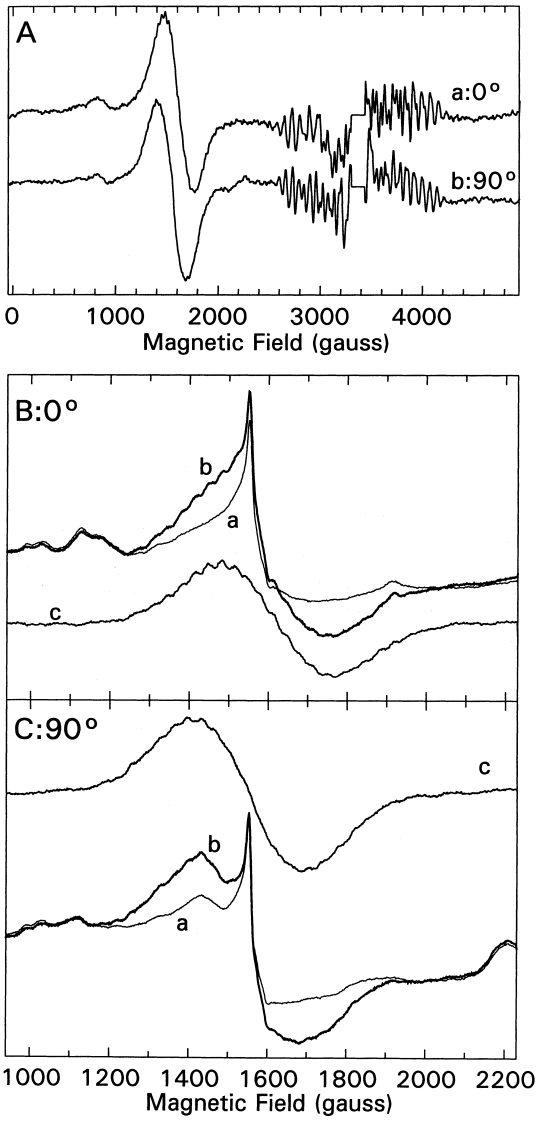

Fig. 5. Orientation dependence of the IR-induced signal in oriented Ca^{2+}-depleted, chelator-treated, polypeptide reconstituted PSII sample. A shows the whole difference spectra (spectra recorded after the IR illumination *minus* that recorded before the IR illumination) with the normal of the membrane parallel (spectrum a) or perpendicular (spectrum b) to the external magnetic field direction. Instrument settings in A: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; temperature, 10 K. The central part of the spectra corresponding to the $Tyr_D^{\bullet}$ region was deleted. B and C show the spectra recorded before (spectra a) and after the IR illumination (spectra b) with the normal of the membrane parallel (B) or perpendicular (C) to the external magnetic field. Spectra c are the difference spectra (spectra b *minus* spectra a). Instrument settings in B and C: modulation amplitude, 10 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; temperature, 10 K. The vertical scales in B and C are different.

dressed by the experiment shown in Fig. 6. Conditions under which the samples were prepared are identical to those in Fig. 1. Spectrum a was recorded in a dark-adapted sample and spectrum b was recorded after illumination at 200 K. Both spectra were recorded at 30 K. The light-*minus*-dark spectrum (spectrum c) exhibits no detectable signal at $g=6$ (i.e. $\approx$1125 gauss). Fig. 1 also shows no evidence for a signal at $g=6$ under other conditions of power and temperature.

It has been suggested that the $g4^{IR}$ signal arises from an $S=5/2$ $Mn^{IV}Mn^{IV}X^{\bullet}$ structure where $X^{\bullet}$ is an organic radical [24,26,27]. An EPR signal attributed to an excited state of this radical was reported in samples measured between 15 and 30 K. The existence of such a radical signal has been investigated here (Fig. 7). Conditions under which the sample was prepared are identical to those in Fig. 2. In Fig. 7A and B, the spectra a were recorded after illumination at 200 K. Spectra b were recorded after a further IR illumination at 150 K. In Fig. 7A, spectrum c is the difference spectrum (spectrum b *minus* spectrum a) showing the formation of the $g4^{IR}$ signal with the corresponding disappearance of the multiline signal. The spectra in Fig. 7B were recorded at 30 K and with instrument settings similar to those reported for the detection of the $X^{\bullet}$ radical [24,26,27]. Fig. 7B shows that no radical is detected at 30 K when the $g4^{IR}$ signal is present.

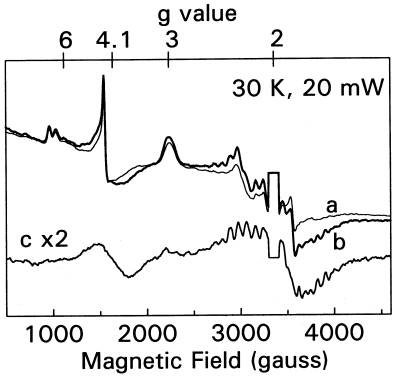

Fig. 6. EPR spectra recorded in dark-adapted PSII (spectrum a) and after a 200-K illumination (spectrum b) in a sample containing 0.5 M sucrose and no alcohol. Spectrum c corresponds to the light-*minus*-dark spectrum. The amplitude of spectrum c was multiplied by the indicated factor. The central part of the spectra corresponding to the $Tyr_D^{\bullet}$ region was deleted. Instrument settings: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; temperature, 30 K.

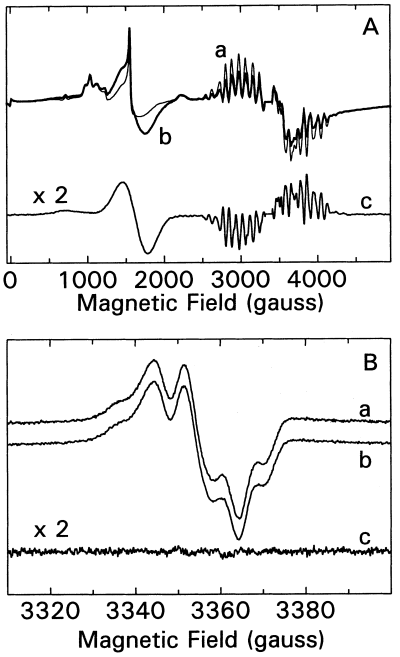

Fig. 7. EPR spectra recorded after 200 K illumination in a sample containing 0.3 M sucrose and ethanol. Spectra a were recorded after the 200 K illumination and spectra b were recorded after a further IR illumination given at 150 K. Spectra c corresponds to the IR-induced spectra. They are multiplied by the indicated factor. Instrument settings in A: modulation amplitude, 25 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; temperature, 10 K. Instrument settings in B: modulation amplitude, 2.8 gauss; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; microwave power, 0.5 μW; temperature, 30 K.

4. Discussion

In the S_2 state, two EPR signals at $g=4.1$ (or $g=4.25$) can be distinguished in terms of their method of generation and their stability: the $g4^{IR}$ signal is generated by IR illumination between $\approx$77 and $\approx$160 K of the spin 1/2 multiline state, the $g4^{S}$ signal can be formed under the same conditions as the spin 1/2 multiline signal (i.e. by illumination at temperatures ranging from physiological temperature down to around 120 K). In the present work, we have compared these two signals in order to test if they originate from the same or from different chemical origins.

We found that the microwave power saturation properties of the two signals measured at 4.2 K were virtually identical. Furthermore, their temperature dependencies measured at non-saturating powers were also identical. The presence of Curie law behavior for the $g4^S$ and $g4^{IR}$ signals indicates that the states responsible for both signals are ground states. These findings are best interpreted assuming that the two signals have a common origin.

The finding that the $g4^{IR}$ signal arises from a ground state agrees with several earlier reports (see Table 1). The finding that the $g4^S$ signal is also a ground state agrees with an earlier report (see Table 1), but disagrees with more recent data showing non-Curie behavior for this signal and indicating its origin from an excited state [24–27]. We cannot explain the origin of this discrepancy. For want of any obvious explanation, we can note that the signal-to-noise ratio of the spectra shown in the previous study was rather poor. The excited state origin for the $g4^S$ signal was a key argument behind the suggestion that the two types of $g4$ signals had different origins. The absence of evidence for the $g4^S$ being an excited state seriously questions that suggestion.

In the present study we also looked at a $g4^{IR}$ signal (in Ca^{2+}-depleted/chelator-treated PSII) in oriented samples. The orientation dependence, anisotropy and resolved hyperfine structure of the $g4^{IR}$ signal were found to be virtually indistinguishable from those reported earlier for $g4^S$ signals [28,29,35]. These marked similarities also argue for a common chemical origin for the two types of signal. Earlier studies reported quite different g-anisotropy for the $g4^{IR}$ and $g4^S$ signals when measured using Q-band spectroscopy [25–27]. We have no explanation for this discrepancy other than that proposed by the authors themselves, i.e. there are some subtraction artifacts in the Q-band spectra [27]. Clearly more studies using Q-band EPR would help to resolve this discrepancy with the present work.

Some earlier published data on the $g4$ signals are also relevant to the present discussion. The replacement of Ca^{2+} by Sr^{2+} results in a shift in the g-value of the $g4^S$ signal from $g=4.1$ to $g=4.25$ [22]. We showed earlier that in an Sr^{2+}-reconstituted sample where the $g4^S$ signal is eliminated by the addition of ethanol, the $g4^{IR}$ signal generated in the same sample showed the same shift in g-value as seen in the $g4^S$ signal [16]. This result is also in favor of a common chemical origin for the two $g4$ signals.

The model in which the two $g4$ signals arise from different chemical species received support from two other lines of experimental evidence [24–27]. First, for the spin system responsible for the $g4^S$ signal, (attributed at that time to an excited state but see above), a signal at $g=6$ was favored at around 30 K and this was proposed to be a higher excited state (5/2 state) which was thermally accessible at this temperature. We were unable to find evidence for the $g=6$ signal.

The absence of a phenomenon (particularly an EPR signal) is a very difficult thing to demonstrate definitively as it can always be argued that the signal was missed for a number of reasons. However, we note that signals at around $g=6$ can arise from O_2 (unless O_2 is specifically eliminated from the sample), from intrinsic and extrinsic Fe^{3+} and from ground state spin 5/2 signals from S_2 under certain conditions and thus there is plenty of scope for confusion in this spectral region. Given this situation and our failure to find a $g=6$ signal related to the $g4^S$ state, we think it is reasonable to question the existence of

Table 1
Characteristics of the 94 signals

	Spin state[a]	Ground/excited state	References
$g4^S$	3/2	ground state	[23]
$g4^S$	3/2	excited state	[24–27]
$g4^S$	5/2 or 3/2	ground state	[28,29]
$g4^S$	5/2	ground state	[30], this work
$g4^S$+$g4^{IR}$	5/2	not determined	[31]
$g4^{IR}$	3/2	ground state	[8,32]
$g4^{IR}$	5/2	ground state	[26,33]

[a]Proposed spin state values from direct experiments or discussion on the data in the literature.

this state, even though we cannot completely rule out its existence. In any case, the presence of a thermally accessible state above the state responsible for the $g4^S$ state does not in itself argue for or against separate chemical origins for the two types of $g4$ signals. The important point in the present study is that no such state is detectable in samples containing either the $g4^S$ or the $g4^{IR}$ signals and thus, from our data, there is no evidence for the two signals arising from different chemical origins.

The second and more important piece of spectroscopic evidence supporting the model where the two forms of the $g4$ signal have different origins is the report of a radical signal at 30 K in samples containing the $g4^{IR}$ signal [25–27]. The rationale that led to the report of this signal was as follows: the $g4^{IR}$ arises from a ground state (see above) and in the two-site model, this was proposed to arises from a Mn dimer. In order to obtain a ground state $g4$ signal from a coupled dimer of Mn, the existence of a third spin had to be invoked. Thus it was proposed that the $g4^{IR}$ signal arose from a $Mn^{IV}M^{IV}X^{\bullet}$ state, where $X^{\bullet}$ is an organic radical, possibly an amino acid radical [25–27]. In the present work, we found no evidence for such a radical signal under these conditions.

Once again, care must be taken in trying to prove the absence of a signal; however, in this case, we can argue clearly that our experimental approach is better in several respects and we can also provide a specific rationale to explain the earlier data. In the present study, we not only had a better signal-to-noise ratio, but also had much better control over the chemistry that was occurring. Here we generated the S_2 states in all centers by illumination at 200 K, then lowered the temperature to a temperature appropriate for the IR-induced conversion of the spin 1/2 state to the state responsible for the $g4^{IR}$ signal. This gives not only a high yield of the $g4^{IR}$ signal (in $\approx$50% of the reaction centers), but, importantly, it also eliminates any side-path photochemistry. The earlier work used broad-band illumination at 130 K which results in S_2 formation in only a fraction of the centers ($\leq$30%, [15]) and a fraction of this (i.e. $\approx$50% of $\leq$30%) is then converted to the $g4^{IR}$ state through the inadvertent presence of IR light. In the centers where S_2 is not formed, other electron transfer reactions take place leading to either oxidation of cyt b_{559}, chlorophyll or carotenoid. The two latter reactions give rise to free radical formation ([39] and references therein). This is another potential cause of problems since such radicals can decay upon warming of the samples at 200 K. Indeed, from close examination of the data in [25,26], it appears that about 50% of the radical signal which disappeared upon warming at 200 K is similar to an EPR signal from a chlorophyll or carotenoid cation radical. The remaining fraction appears to be attributable to $Tyr_D^{\bullet}$ which may be undergoing changes in relaxation. Given these arguments and our inability to detect a radical associated with the $g4^{IR}$ signal, we consider it reasonable to question the existence of such a radical.

Overall, then, we have obtained several lines of evidence indicating that both forms of the $g4$ EPR signal arise from the same species. We have been unable to confirm the behavior reported earlier indicating that the $g4^S$ is an excited state, nor were we able to confirm the presence of signal from a higher excited state in samples containing the $g4^S$, nor a radical signal in samples containing the $g4^{IR}$. We conclude, then, that at present, a common origin for both types of $g4$ signal is the best explanation for the data. We should add that in the majority of earlier studies, it was at least implicitly assumed that the two forms of the $g4$ signal arose from the same chemical species. The conclusion drawn here is in line with that earlier assumption.

The hyperfine structure seen on the $g4^S$ signal was interpreted as a strong line of evidence for a magnetic tetranuclear Mn origin for this signal [28,29]. Our present data showing the same hyperfine structure in the $g4^{IR}$ signal are in agreement with this proposal and extend it to the $g4^{IR}$ signal. In this previous study [28,29], the hyperfine structure in the $g4^S$ signal was only observed in the presence of ammonia. In native PSII, we have not found conditions under which the hyperfine structure can be observed in the g_4^{IR} signal. Nevertheless, observation of the same hyperfine structure in the $g4^{IR}$ signal generated in Ca^{2+}-depleted, EGTA-treated PSII and in the $g4^S$ signal generated in ammonia-treated PSII is probably not coincidental. This certainly reveals that the hyperfine structure arises from an intrinsic structural property of the Mn-cluster and does not result from a chemical perturbation.

Earlier, we concluded that the $g4^{IR}$ signal arises from a spin 5/2 state based on a SQUID magnetization study [33]. At that time, given the existence of the model suggesting two different origins for the $g4$ signal, we were unable to extend our conclusions to the $g4^{S}$ signal. Nevertheless, Haddy et al. [30] had already concluded that the $g4^{S}$ signal arose from a 5/2 state based on simulations of the signals obtained at several microwave frequencies. Given the present results, we consider that the data from Haddy et al. [30] and our own [33] concluding both to a spin 5/2 state at the origin of the $g4^{S}$ and $g4^{IR}$ signals, respectively, are in agreement with each other.

Also in agreement with the proposition that an $S=5/2$ state is at the origin of the $g4$ signals is a recent report in which a pseudo tetrahedral $Mn^{III}(Mn^{IV})_3$ complex has been synthesized [40]. This complex presents a spin topology in which the 4 Mn ions are magnetically coupled and exhibits an $S=5/2$ ground state with an EPR signal at $g=4.1$. Moreover, the D value (1.1 cm^{-1}) corresponding to this complex and measured in a SQUID magnetization study is close to that of the $g4^{IR}$ signal in PSII determined in the same kind of experiment [33].

An $S=5/2$ value was also proposed for the state giving rise to the $g=4.1$ signal from a pulsed-EPR study [31] done on a $g4$ state generated by an illumination at 130 K. Since the sample contained sucrose, the S_2 state which was formed in these conditions is expected to exhibit both the $g4^{IR}$ and $g4^{S}$ signals. Nevertheless, the reported light-*minus*-dark spectrum was obtained by recording the dark spectrum after a dark adaptation period at 0°C following the 130 K illumination. In these experimental conditions, changes in the amplitude of signals from contaminating Fe^{3+} (spin 5/2) are frequently observed and thus it seems quite possible that Fe^{3+} signals contribute to the studied spectrum. To eliminate this doubt, the pulsed-EPR study should be done on the signal which is formed at 130 K and reversed by warming to 200 K rather than 0°C.

The idea that the two $g4$ signals were seen as arising from separate Mn dimers was taken as an important factor in a structural model where the Mn cluster is seen as being made up of two separate dimers which are in electron transfer contact [24–27]. Another factor behind this model is that the Mn-multiline-EPR signal in S_2 was simulated as arising from an isolated $Mn^{III}Mn^{IV}$ dimer using unusual quadrupole parameters. There has been some debate over the validity of this model [6–9,41–43], but the 'two separate Mn dimers' model has gained credibility recently (e.g. [44–47]). The present paper, which not only questions several of the main lines of evidence that led to the assignment of the two types $g4$ signals to two separate chemical species, but also presents several arguments in favor of a common chemical origin, thus weakens the case for the two separate dimers model. The majority of researchers in the field seem to consider that the best explanation of the S_2 spin 1/2 multiline signal is that it arises from a cluster of four magnetically coupled Mn ions [6–9,41–43]. While this model remains to be definitively proven, it is still the most reasonable, given the existing data.

Some other EPR data were explained by a model implying electron transfer within the Mn_4-cluster. Indeed, by using parallel polarization EPR detection, a signal with a turning point at $g=4.8$–4.9 [48,49] has been attributed to the fraction of the Mn-cluster in the S_1 state which exhibits the S_2-multiline signal upon oxidation by illumination at low temperature. In this model, the $g=4.1$ signal formed by illumination at 130 K arose from a distinct magnetic species. Conversion of the $g=4.1$ signal into the multiline S_2-signal upon warming to 200 K was interpreted as the reduction of the species exhibiting the $g=4.1$ signal by that exhibiting the $g=4.8$ signal. To reconcile this model with the finding that the $g=4.1$ signal results from the effect of IR light on the S_2-multiline state, we must imagine that IR illumination of the S_2-multiline state induces the $g=4.8$ signal in the same centers as those in which the $g=4.1$ signal is formed. Preliminary experiments done by using a parallel mode EPR cavity indicates that no $g=4.8$ signal could be detected, at least between 2 and 8 K, after IR illumination of the S_2-multiline state in a sample similar to that used in Fig. 2 (not shown). At present, therefore, we are unable to reconcile the reported low temperature behavior of the parallel mode signal at $g=4.8$ with the IR effects on the S_2 state.

Although we conclude from the present work that the two forms of the $g4$ signal arise from the same chemical species, this species must still exist in two forms that differ from each other in terms of their stability: the $g4^{IR}$ being unstable and converting to

the S_2-multiline state at temperatures of 200 K and above, while the $g4^S$ state has a stability which is comparable to that of the S_2-multiline state. Consequently, the $g4^{IR}$ and $g4^S$ signals would be expected to exhibit some structural differences, but these have no effect on the physical properties studied in this work.

The states responsible for the $g4^S$ signal and the multiline signal are probably in equilibrium at room temperature with the S_2-multiline state normally being the lower energy state. The various pretreatments that lead to an increase in the proportion of centers giving rise to the $g4^S$ signal in place of the multiline signal presumably stabilize the $g4^S$ state relative to that of the multiline state. On the other hand, the presence of alcohols destabilize the $g4^S$ state versus the multiline state. From the IR effects, we have suggested that the $g4^{IR}$ and the S_2-multiline states represent the same structure and valence state of the Mn cluster, only differing from each other either in terms of the valence distribution, or in terms of the spin state of the Mn^{III} ion [10,16,33]. The biochemical pretreatments that favor the $g4$ state should then be seen as changing the environment to one that favors the $g4$ state valence distribution (e.g. through electrostatic effects), or changing the coordination around the Mn to favor the different spin state of the Mn^{III} ion.

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