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Accelerated Publications

High-Spin States ($S \geq {}^5/_2$) of the Photosystem II Manganese Complex

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ABSTRACT: The Mn_4 complex which is involved in water oxidation in photosystem II (PSII) is known to exhibit two types of EPR signals in the S_2 state, one of the five redox states of the enzyme cycle: either a multiline signal ($S = {}^1/_2$) or a signal at $g = 4.1$ ($S = {}^3/_2$ or $S = {}^5/_2$). The $S = {}^1/_2$ state can be converted to that responsible for the $g = 4.1$ signal upon the absorption of near-infrared (IR) light [Boussac, A., Girerd, J.−J., and Rutherford, A. W. (1996) *Biochemistry 35*, 6984−6989]. It is shown here that a third state gives rise to signals at $g = 10$ and 6. This state is formed by IR illumination of the $S = {}^1/_2$ state at 65 K, a temperature where IR illumination leads to the loss of the $S = {}^1/_2$ signal but to no formation of the $g = 4.1$ state. On the basis of the corresponding decrease of the $S = {}^1/_2$ state, the new state can be trapped in ~40% of the PSII centers. Warming of the sample above 65 K, in the dark, leads to the loss of the $g = 10$ and 6 resonances with the corresponding appearance of the $g = 4.1$ signal. It is suggested that the IR-induced conversion of the $S = {}^1/_2$ state into the $g = 4.1$ state at 150 K involves the transient formation of the new state. The new state is attributed to a $S = {}^5/_2$ state of the Mn_4 complex (although a S value $> {}^5/_2$ is also a possibility). Spectral simulations indicate an E/D ratio of −0.05 with $|D| \leq 1$ cm^{-1}. The resonances at $g = 10$ and 6 correspond to the g_z of the $\pm{}^5/_2$ and $\pm{}^3/_2$ transition, respectively. The temperature-dependent conversion of this $S = {}^5/_2$ state into the $g = 4.1$ state is proposed to be due to relaxation of the ligand environment around the Mn_4 cluster that leads to a change in the zero field splitting parameters, assuming an $S = {}^5/_2$ value for the $g = 4.1$ state. The new form of the S_2 state reported here may explain some earlier data where the S_2 state was present and yet not detectable as either a $S = {}^1/_2$ or a $g = 4.1$ EPR signal.

Photosystem-II (PSII)[1] catalyzes light-driven water oxidation resulting in oxygen evolution. The reaction center of PSII is made up of two membrane-spanning polypeptides (D1 and D2) analogous to the L and M subunits of the purple photosynthetic bacterial reaction center (see ref *1*, for a review). Absorption of a photon results in a charge separation between a chlorophyll molecule (P_{680}) and a pheophytin molecule. The pheophytin anion transfers the electron to a quinone Q_A and $P_{680}{}^+$ is reduced by a tyrosine residue, TyrZ. A cluster constituted of four manganese ions (Mn_4), located in the reaction center of PSII, probably acts both as the active site and as a charge accumulating device of the water-splitting enzyme. During the enzyme cycle, the oxidizing side of PSII goes through five different redox states that are denoted S_n, n varying from 0 to 4. Oxygen is

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[1] Abbreviations: P_{680}, chlorophyll (Chl) center of photosystem II (PSII); EPR, electron paramagnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PPBQ, phenyl-*p*-benzoquinone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; IR, infrared.

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released during the S_3 to S_0 transition in which S_4 is a transient state (*2*−*5*).

In oxygen evolving PSII, the S_2 state gives rise to EPR signals which are detectable using conventional EPR. The first signal detected from S_2 was a multiline signal near $g = 2$ (*6*). This signal is spread over roughly 1800 G and is made up of at least 18 lines, each separated by approximately 80 G. Currently, the most commonly favored origin for the S_2 multiline signal is that it arises from a Mn tetramer which includes a di-μ-oxo($Mn^{III}Mn^{IV}$) motif (reviewed in refs 2 and 7−9). The second signal attributed to the S_2 state is centered at $g = 4.1$ (*10*−*13*) or $g = 4.25$, depending on experimental or preparation conditions. The $g = 4.1$ signal has been proposed to arise from either a $S = {}^3/_2$ state (e.g., refs 7, 14, and 15) or a $S = {}^5/_2$ state (*16*, *17*). Recently, a different multiline signal has also been observed in the S_0 state (*18*−*20*).

It has been shown that the state responsible for the S_2 multiline signal can be converted to that responsible for the $g = 4.1$ signal upon the absorption of IR light by the Mn_4 cluster itself (*21*). The action spectrum of this conversion has its maximum at 820 nm. The similarity of this action spectrum to the inter-valence charge-transfer band in di-μ-oxo($Mn^{III}Mn^{IV}$) model systems (*22*) led us to suggest that the conversion of the multiline signal to the $g = 4.1$ signal resulted from an electron transfer from Mn^{III} to Mn^{IV} in the Mn^{III}(di-μ-oxo)Mn^{IV} dimer of the Mn_4 cluster. Alternatively, we have also proposed that the valence swapping induced by near-IR light could occur between the two dimers (*23*). A third possibility is that the Mn^{III} in the Mn_4 complex undergoes a spin-state transition from $S = 2$ to 1, resulting in the $g = 4.1$ signal formation (*23*). Such a model is based on precedence with iron photochemistry where illumination converts the Fe^{II} from the low-spin state ($S = 0$) into the high-spin state ($S = 2$), or vice versa, depending the wavelength of the light (*24*, *25*).

Earlier results in the literature, which showed that the $g = 4.1$ state was preferentially formed by illumination at 130 K (*10*), were reinterpreted as being 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 conversion of this state to the $g = 4.1$ state due to the simultaneous and inadvertent presence of 820 nm light in the broad-band illumination given (*21*).

Under some circumstances, the $g = 4.1$ (or $g = 4.25$) signal is stabilized at temperatures equal to or above 200 K (*10*, *12*, *26*−*29*). Clearly, there can be no role of near-IR light in the formation of the signal under these conditions. In this case, the "$g = 4$" state must be stabilized in some way to make it the energetically preferred state.

The yield of the multiline signal to $g = 4.1$ (or $g = 4.25$) signal conversion was found to be maximum at 150 K in untreated PSII and at 100 K in Ca^{2+}-depleted, EGTA-treated PS II (*21*) and decreased below these temperatures. Two models were proposed to explain the low-temperature limit (*21*): (i) thermal energy is required to overcome an activation barrier between unstable intermediates; (ii) the Mn_4 structure responsible for the $g = 4.1$ and $g = 4.25$ signals is formed from the effect of near-IR light on an excited state of the Mn_4 structure rather than on the ground state which is responsible for $S = {}^1/_2$ S_2 multiline signal.

In this report, the effect of near-IR illumination below 150 K on the Mn_4 cluster has been investigated. It is shown that, below 150 K, IR light converts the Mn_4 cluster into a state which exhibits characteristic EPR signals from a $S = {}^5/_2$ state.

MATERIALS AND METHODS

Untreated oxygen evolving PSII-enriched membranes from spinach and Ca^{2+}-depleted, EGTA-treated, and polypeptides-reconstituted PSII were prepared as previously described (*30*). Sr^{2+}-reconstituted PSII membranes were prepared as in ref *27*. Untreated PSIIs were submitted to one additional washing in 0.3 M sucrose, 25 mM Mes, pH 6.5, 10 mM NaCl, and 0.1 mM EDTA.

The PSII preparations were put in quartz EPR tubes (at 6−8 mg of Chl/mL) and dark-adapted for 1 h, at 0 °C. For, the oxygen-evolving PSII preparation, PPBQ, dissolved in ethanol 95% (Carlo Erba), was added as an artificial electron acceptor. After the addition of PPBQ, the samples were immediately frozen in the dark at 200 K in a carbon dioxide-ethanol bath. Then, the samples were degassed before to be transferred to liquid nitrogen (77 K). Degassing of the sample was done in the dark at 200 K. At this temperature O_2 is easily removed (as estimated by magnetic susceptibility measurements, not shown) from the sample by pumping the EPR tube on a vacuum line (~10^{-2} mbar). The sample, always at 200 K, was then placed under a He_2 atmosphere. After such a procedure was repeated 3 or 4 times, the EPR tube was transferred to 77 K. This protocol avoids the problems linked to the use of the classical freeze−thaw procedure with concentrated membranes (i.e., inhibition of the sample and bubbling during the thawing phase). CW-EPR spectra were recorded at liquid helium temperatures with a Bruker ESP300 X-band spectrometer equipped with an Oxford Instruments cryostat.

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 (which absorb above 750 nm) in a nonsilvered dewar at 200 K (ethanol, solid $CO_{2)}$. Above 80 K, near-IR illumination of the samples was done in a nitrogen gas flow system (Bruker, B-VT-1000). At and below 65 K, IR illumination of the sample was done in the EPR cavity. 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. In all the conditions tested, the duration of the near-IR illumination (60−90 s) was adjusted to produce the spin state conversion in the maximum amount of reaction centers.

RESULTS

IR illumination at various temperatures was first performed in the S_2 state of oxygen evolving PSII. Spectrum a in Figure 1A was recorded after illumination at 200 K of a dark-adapted oxygen evolving PSII. This spectrum exhibits the characteristic EPR multiline signal (between 2400 and 4200 G) which arises from the Mn_4 complex in the S_2 state. Also visible are (i) the g_z signal of cytb$_{559}$ (at 2250 G), which is oxidized in a fraction of the centers; (ii) a broad signal underlying the multiline signal at ~3600 G ($g = 1.9$) and which originates from Q_A-Fe^{2+}; and (iii) below 2200 G,

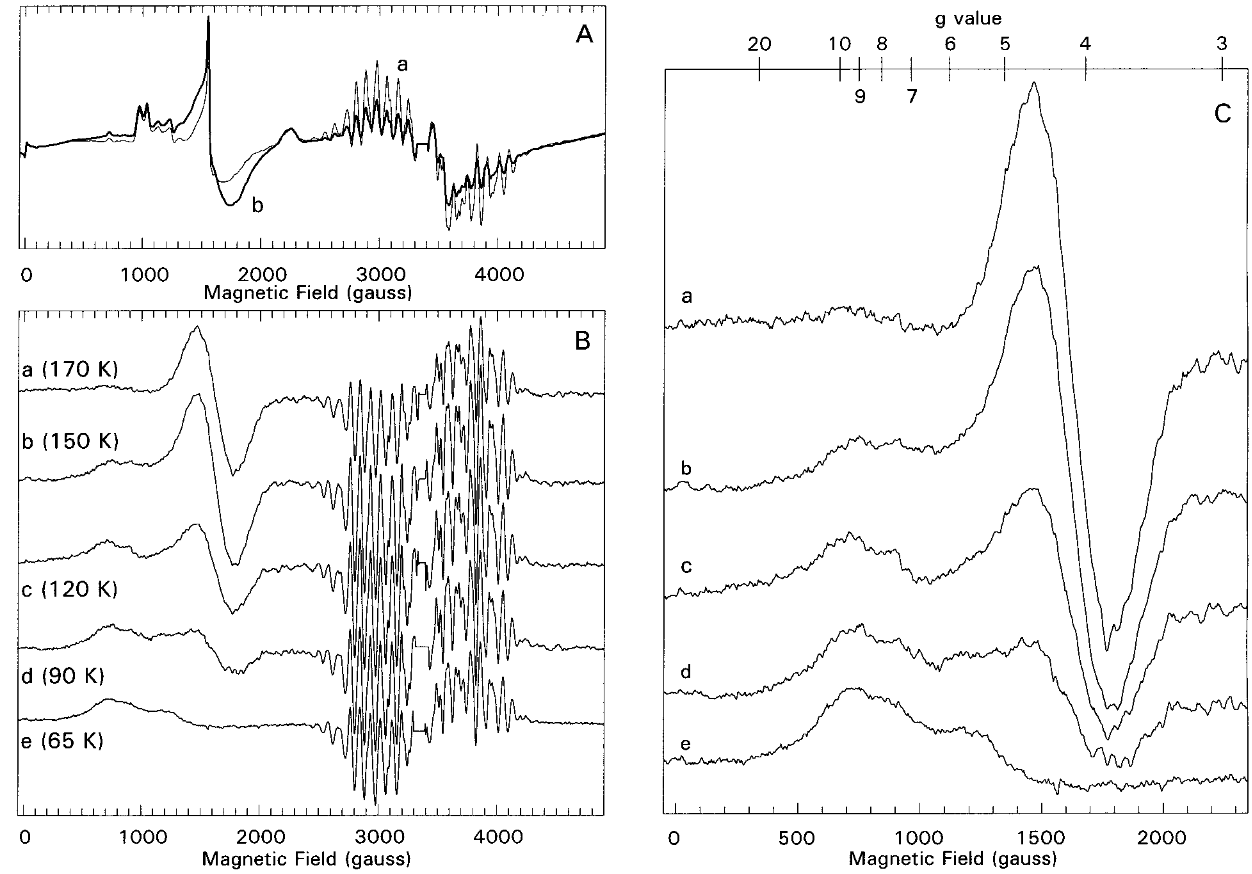

FIGURE 1: (A) EPR spectra of untreated PSII recorded at 10 K after a 200 K illumination (spectrum a) and after a further 813 nm illumination given at 150 K (spectrum b). (B) Shows the spectra obtained by subtracting the spectrum recorded before the IR illumination from that recorded after the IR illumination. The 813 nm illumination was given at 170 K, spectrum a; at 150 K, spectrum b; at 120 K, spectrum c; at 90 K, spectrum d; at 65 K, spectrum e. The number of accumulations for spectrum e was double that for the other spectra, and therefore, its amplitude was divided by two to be comparable to the other spectra. (C) Same spectra as in panel B scaled to the same multiline signal amplitude as estimated by a double integration. Instrument settings: 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.

signals from contaminant high-spin Fe^{3+}. Normally, in the presence of sucrose, illumination at 200 K to form the S_2 state, induces a $g = 4.1$ signal in a fraction of the centers at the expense of the multiline signal (*12*, *13*). To prevent this $g = 4.1$ formation, i.e., to maximize the proportion of centers in which the multiline signal was present before the near-IR illumination was given, 2−3% ethanol was added to the sample (*13*). Indeed, no $g = 4.1$ signal is detected in spectrum a of Figure 1A.[2]

Spectrum b in Figure 1A was recorded after a further near-IR illumination at 150 K following the 200 K illumination. The difference spectrum, spectrum b minus spectrum a in Figure 1A, is shown in Figure 1B (spectrum b). The signals which disappear upon IR illumination appear negative, and the IR-induced signals appear positive. As reported earlier (*21*), IR illumination converted the state responsible for the multiline signal into a state responsible for the $g = 4.1$ signal (~1600 G).

Figure 1B also shows the results from an experiment in which the PSII sample in the S_2 state was illuminated by near-IR light at different temperatures (from 170 to 65 K). Two observations can be made. First, the yield of the spin conversion (as estimated by the amplitude of the multiline signal which disappeared) is maximum at 150 K (*21*) and decreases above and below this temperature. Second, the shape of the signal induced by the near-IR light varies considerably depending on the temperature at which the IR illumination was given. Figure 1C shows, on an expanded magnetic field scale, the near-IR-induced EPR signals above $g = 3$. Amplitudes of the spectra in Figure 1C are normalized so that they correspond to the same amount of IR-susceptible multiline signal (as estimated by double integration of the multiline signals in Figure 1B). The principal effect of IR illumination performed at 170 K (spectrum a) was to induce a derivative signal centered around 1600 G ($g = 4.1$). A very small resonance at around 700 G ($g \approx 10$) is also detectable. At temperatures below 170 K, the yield of the $g = 4.1$ signal formation decreased while the signals between 500 and 1500 G increased. When the IR illumination was performed at 65 K, no $g = 4.1$ signal was induced (spectrum e). Spectrum e only exhibits resonances at $g \approx 10$ and 6. These resonances are characteristics of a state $S = {}^{5}/_{2}$ (see below). Therefore, it

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[2] It has been reported that ethanol modifies the saturation curve [amplitude versus (microwave power)$^{1/2}$] of the multiline signal (*13*). This effect is probably due to a contaminant in the absolute ethanol previously used since it is not observed with ethanol (95%) (not shown).

[3] The signals at $g \approx 10$ and $g \approx 6$ are also observable in PSII preparations containing no sucrose in the medium or using dimethyl sulfoxide instead of ethanol as a solvent for PPBQ. The most important technical point which influences the easiness with which they are detected is the reproducibility of the baseline before and after the IR

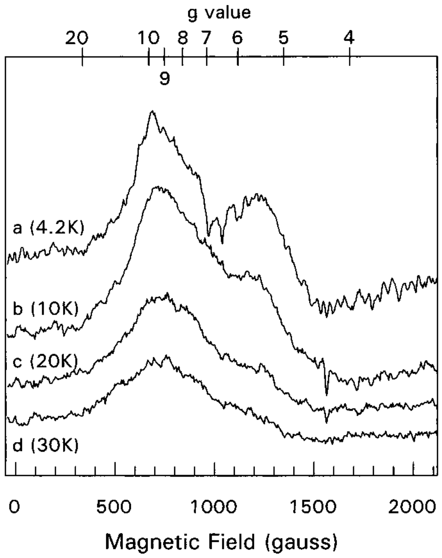

FIGURE 2: Spectra recorded on untreated PSII and obtained by subtracting the spectrum recorded before the IR illumination from that recorded after the IR illumination given at 65 K. The EPR spectra were recorded at 4.2 K (spectrum a), at 10 K (spectrum b), at 20 K (spectrum c), at 30 K (spectrum d). Same instrument settings as in Figure 1.

appears that, at 65 K, a 813 nm illumination of the Mn$_4$ cluster converts its spin state from the $S = {}^1/_2$ value characterized by the multiline signal into a $S = {}^5/_2$ value characterized by signals at $g \approx 10$ and 6.[3]

Figure 1B shows that the yield of the IR-induced spin transition at temperatures below 150 K is significantly higher than that previously observed (*21*). This difference could be due to a kinetic effect because the intensity of the IR light used in the present work (a 813 nm laser diode) is considerably higher than in the previous study (broad-band illumination given with a tungsten lamp). In Figure 1, the yield of the spin transition at 65 K corresponds to 60−70% of the yield observed at 150 K. At temperatures below 65 K, the yield of the spin conversion gradually decreased and at 10 K it attained about 30−35% of the yield observed at 150 K. The high spin signals formed at 10 K are similar to those induced at 65 K (not shown).

Illumination of the sample at 65 K with a 800 W tungsten lamp through a Corion filter which absorbs light below 910 nm induced no detectable $g > 5$ signals. Illumination of the sample at 65 K with a 800 W tungsten lamp through water (which absorbs above 900 nm) and IR filters which absorbs light above 750 nm induced a mutiline to $g > 5$ signals conversion in less than 10% of the reaction centers (not shown). These results indicate that the action spectrum of the S$_2$ multiline signal to $g > 5$ signals transition is similar to that of the S$_2$ multiline signal to $g = 4.1$ signal transition (*21*).[4]

In metallic systems with $S = {}^5/_2$ (and a small D value), the relative amplitudes of the resonances at $g > 4$ are expected to be strongly temperature dependent. Figure 2 shows a preliminary study of the temperature dependence of the signals induced at 65 K by IR light. As expected, the amplitude of the $g \approx 10$ and 6 resonances varies significantly with the temperature at which they are recorded (from 4.2 to 30 K).

IR illumination has been shown to convert the state responsible for the multiline signal into the state responsible for a $g = 4$ signal in oxygen evolving PSII and also in Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII and Sr^{2+}-reconstituted PSII (*21*). By contrast with intact PSII and Sr^{2+}-reconstituted PSII in which the maximum spin conversion occurred at 150 K, the maximum IR-triggered spin conversion in the S$_2$ state of the Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII occurred at 100 K. Therefore, we also studied the effect of near-IR light onto the modified PSII preparations at temperatures lower than 100 K.

Figure 3 (spectrum b) shows the effect of near-IR illumination at 50 K of a Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII. Spectrum b can be compared to that obtained with untreated PSII (spectrum a). In Figure 3, the amplitude of spectrum b has been normalized to that of spectrum a so that the double integration of the multiline signals are equivalent. Therefore, the EPR signals in spectra a and b correspond to the same reaction center concentration. Spectrum b in Figure 3 is also characteristic of a $S \geq {}^5/_2$ state with a strong resonance at $g \approx 9$ with a shoulder at a higher g value (see below).

Near-IR illumination of Sr^{2+}-reconstituted PSII at 65 K induced signals similar to those detected in spectrum b of Figure 3. We have shown previously that IR illumination at higher temperatures converts the state responsible for the multiline signal into the state responsible for a $g = 4.25$ signal both in Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII and Sr^{2+}-reconstituted PSII (*21*). This suggests that, in these two PSII preparations, the structural differences in the Mn$_4$ cluster, which are at the origin of the two different S$_2$ multiline signals (the spectral differences and the difference in stability), are not manifest in the spectra of the high-spin states of the manganese complex.

The spectrum induced by IR light in untreated PSII, at 65 K, (Figure 3, spectrum a) was simulated using the first-order effective g values previously described (*31*−*33*) and transition probabilities derived by Holuj (*33*). The effective g values were calculated from the eigenvalues of the zero-field Hamiltonian listed by Wickman et al. (*32*). These eigenvalues are dependent on a single parameter, the E/D ratio. For a single value of E/D, three sets of $\mathbf{g}$ tensors are obtained corresponding to the transitions within each of the three Kramer's doublets. In principle, from the temperature dependence of a spectrum, it should be possible to obtain the value of D and its sign. The calculated powder spectra

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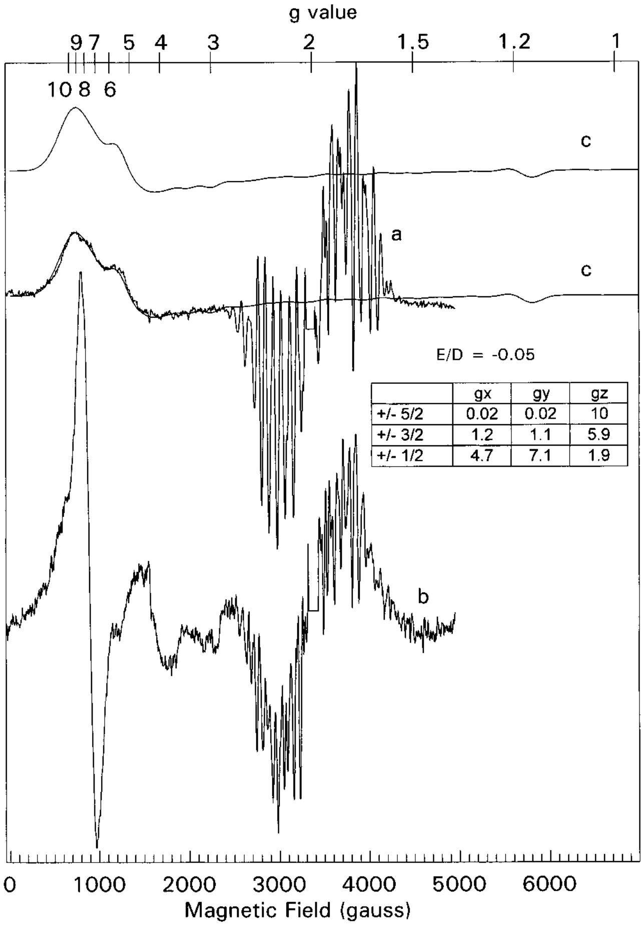
illumination. Good reproducibility required the removal of oxygen from the EPR tube. Oxygen does not prevent the formation of these signals but small changes in its EPR signal (intense at $g \approx 5$) decrease considerably the quality of the spectra.

[4] Considering the absorption of water and that of the chlorophyll, a reviewer questioned the validity of the action spectrum given in (*21*). The peak at 820 nm is nevertheless very probably real. Indeed (i) below 900 nm, absorption of H$_2$O is weak, (ii) experiments performed in D$_2$O (which absorbs at longer wavelengths than H$_2$O does) indicate that 820 nm is also the best wavelength to induce the spin transition (not shown), and (iii) with $\lambda \geq 1$ μm or $\lambda \leq 700$ nm, irradiation for a long time and with a strong intensity (i.e., in conditions in which we expect that at least some photons escape absorption by water and Chls) does not induce a Mn photochemistry.



	gx	gy	gz
+/- 5/2	0.02	0.02	10
+/- 3/2	1.2	1.1	5.9
+/- 1/2	4.7	7.1	1.9

FIGURE 3: Spectrum a is identical to spectrum e in panel B of Figure 1 and corresponds to untreated PSII. Spectrum b has been recorded on Ca^{2+}-depleted/EGTA-treated/polypeptides-reconstituted PSII. To obtain spectrum b, the IR illumination was given at 50 K. Same instrument settings as in Figure 1. Both spectra are normalized so that the amplitude of the multiline signals are equivalent as estimated by double integration. Spectrum c, simulated spectrum (see the text). The inset shows some of the parameters used in the simulation. In PSII samples, we could not detect the high field turning point present at ~5900 G in the simulated spectrum.

were obtained by summing the contribution from 10^5 to 10^6 random orientations of the magnetic field relative to magnetic axes systems. Intermediate E/D eigenvalues and energies were determined by polynomial interpolation of the published values (*32*). The resulting spectra were convolved with derivative Gaussian functions.

The outstanding features of the difference spectra in Figure 3 (spectra a and c) are the resonances at $g = 10$ and 6. The former corresponds to the g_z value of the $-^5/_2$ to $+^5/_2$ transition and the latter the g_z value of the $-^3/_2$ to $+^3/_2$ transition. The fact that the $g = 10$ feature is the dominant feature at all temperatures (Figure 2) indicates that the $\pm\,^5/_2$ Kramer's doublet is the lowest state, and hence, the zero-field splitting parameter must be negative. The combination of g values of 10 and 6 is indicative of a small E/D value. A reasonable agreement with the experimental spectra was obtained for a E/D value of -0.05 (Figure 3). The correct relative amplitudes were obtained by manually adjusting the contribution of each of the three **g** tensors. We were unable to reproduce the observed temperature dependence (Figure 2). This may be due to the temperature dependence of the relaxation times, in particular T_2. Such effects have been observed for Fe(III) in ferrichrome A (*32*). This system has a E/D ratio of 0.25 and D a value of -3 cm^{-1}. The ground-state signal with g values of 9.3, 1.7, and 3.0 was only observed at 1.7 K. At 4.2 K, only the transitions from the upper Kramer's doublet were observed, while at 77 K and above, only the $g = 4.3$ transition, arising from the middle Kramer's doublet, was detected. Under such circumstances where relaxation effects strongly effect the spectrum, a simple Curie Law analysis is not possible. However, the presence of a $g = 6$ signal at 4.2 K can be used to obtain an upper-bound on D. The relative energy difference between the two lowest Kramer's doublets is $3D$ and the thermal energy at 4.2 K is approximately 3 cm^{-1}. Since the $g = 6$ signal is clearly visible, $|D|$ must be ≤ 1 cm^{-1}. Paradoxically, at 30 K, neither the $g = 6$ feature nor a $g = 7$ feature, which would result from the highest Kramer's doublet, is observable. This strongly suggests that the variation in amplitude of the resonances arising from the upper two Kramer's doublet is determined by relaxation effects rather than thermal population.

Spectrum b (Figure 3) recorded on the Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII exhibits a small $g = 4.25$ signal. By analogy with untreated PSII, this could suggest that a temperature lower than 50 K would be required to prevent the formation of the state responsible for the $g = 4.25$. Unfortunately, despite the yield of the spin conversion at 100 K being greater in the Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII than in intact PSII at 150 K (*21*), its yield at 50 K is considerably smaller (only ~10% of the multiline signal disappeared). This explains why the signal-to-noise ratio of spectrum b in Figure 3 is lower than the signal-to-noise ratio of spectrum a. This also explains why we have yet to obtain data at lower temperatures than 50 K in such samples. Spectrum b of Figure 3 is more difficult to simulate. Assuming a $S = {}^5/_2$ state, preliminary attempts indicate that the $g = 4.25$ resonance could be associated to the resonance at $g \approx 9$. However, we have not ruled out other possibilities.

We have shown previously that the effects of IR illumination at 150 K (i.e., the multiline to $g = 4.1$ conversion) were reversed by raising the temperature to 200 K (*21, 23*). In untreated PSII, we have now measured the half-time for the recovery of the multiline signal after IR illumination as being about 300 s at 150 K and 30−60 s at 170 K. The temperature dependence of the kinetics of the $g = 4$ signal(s) to the S_2 multiline signal(s) transition was studied in various PSII preparations (not shown). We found no effect of H_2O/D_2O and Cl^-/Br^- exchanges. This suggests that the rate-limiting step in the $g = 4$ signal to multiline signal transition does not involve H^+ or Cl^- displacement.

The new IR-induced signals at $g \approx 10$ and 6 also disappeared and the multiline signal was restored after the samples were incubated few seconds at 200 K (not shown). Because $g > 5$ type signals are formed at lower temperatures than the $g = 4$ type signals, we tested whether the conversion of the $g > 5$ type signals back to the multiline signal occurs via a route which involves an intermediate state giving rise to the $g = 4$ type signal(s).

Figure 4 shows the result of an experiment in which the untreated PSII (spectrum a) and the Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII (spectrum d) were first illuminated at 65 or 50 K, respectively. Then, the

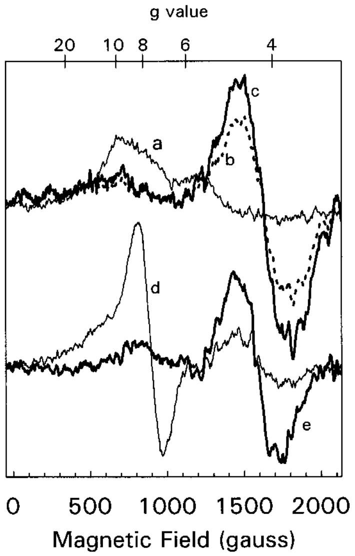


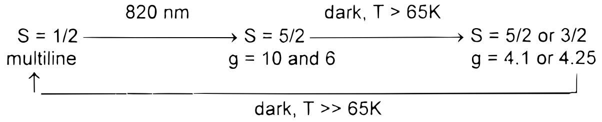
FIGURE 4: Spectra a, b, and c were recorded with untreated PSII. Spectra d and e were recorded with Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII. Spectra a and d were obtained by subtracting the spectrum recorded before the IR illumination from that recorded after the IR illumination given at 65 K (spectrum a) or 50 K (spectrum d). Spectra b and e were obtained by subtracting the spectrum recorded before the IR illumination from that recorded after a dark incubation for 45 s at 170 K (spectrum b) or 100 K (spectrum e) following the IR illumination. Spectrum c corresponds to spectrum b multiplied by a factor 1.5 (see the text). Same instrument settings than in Figure 1.

samples were incubated for 45 s in the dark at 170 K (spectrum b) or 100 K (spectrum e). Spectra b and e are obtained by subtracting the spectra recorded before the near-IR illumination from that recorded after the dark incubation at 170 or 100 K. Figure 4 shows that the $g = 4$ type signals were formed in the dark at the expense of the $g > 5$ type signals. For untreated PSII, the dark incubation at 170 K also resulted in the restoration of the multiline signal in ~35% of the population of the reaction centers which were converted to the higher spin states by the IR light (not shown).Therefore, the number of reaction centers which are responsible for spectrum b in Figure 4 is smaller than the number of those responsible for spectrum a. To compensate for this, spectrum b in Figure 4 has been multiplied by a factor of 1.5 to obtain spectrum c which now corresponds to the same number of reaction centers as in spectrum a. The results in Figure 4 show that, upon raising the temperature, the Mn_4 state responsible for the $g > 5$ type signals converts to a state responsible for a $g = 4$ type signal, and the $g = 4$ state decays to form the state responsible for the $S = {}^1/_2$ multiline signal.

DISCUSSION

The results presented in this work show that the state responsible for the S_2 multiline EPR signal in PSII is converted to a state responsible for EPR signals with g values above $g = 5$ by excitation with near-IR radiation given at a temperature $\leq$ 65 K. The IR-induced signals at 65 K are characteristic of resonances arising from a state $S = {}^5/_2$. Spectral simulations are shown in Figure 3 and indicate a negative D value with E/D ratio of -0.05 with $|D| \leq 1\ \mathrm{cm}^{-1}$. The resonances at $g = 10$ and 6 correspond to the g_z of the $\pm{}^5/_2$ and $\pm{}^3/_2$ transition, respectively.

Although in the present case we have not obtained an accurate action spectrum, tests using light of different qualities indicate that the action spectrum of the S_2 multiline signal to $g = 4.1$ signal conversion and that of the S_2 multiline signal to $g > 5$ signals conversion are identical. In addition, we have shown that the new state decays to the state responsible for the $g = 4$ type signal and that the latter decays to form the $S = {}^1/_2$ state responsible for the multiline signal. We summarize the (photo)chemical reactions of the Mn cluster in the following simplified scheme.



In our earlier work (*21*) on photochemistry of the Mn cluster in PSII where we reported the multiline/$g = 4$ interconversion, we suggested the existence of a IR-induced state formed prior to the $g = 4$ state. The present work confirms that suggestion. Absorption of radiation at $\approx$ 820 nm by the Mn_4 cluster induces a transition between a $S = {}^1/_2$ state into a $S = {}^5/_2$ state irrespective of the temperature at which the sample is illuminated. At temperatures equal to or lower than 65 K, the $S = {}^5/_2$ state is trapped. At temperatures higher than 65 K, an activation barrier between the $S = {}^5/_2$ state and the state responsible for the $g = 4.1$ signal can be overcome and the next stable state becomes that responsible for the $g = 4.1$ signal (see Figure 4).

In the literature, there are few model compounds that have a Mn_4 cluster in the $S = {}^5/_2$ state. A $Mn^{II}Mn^{III}{}_3$ model compound (i.e., a complex with manganese ions in different redox states than those in PSII) with a $S = {}^5/_2$ state and with EPR signals at g values $>$ 5 has been recently synthesized (*34*).

From the model presented above, no conclusion can be made on the spin value of the state responsible for the $g = 4.1$ signal. In spectrum a of Figure 1C, a small signal at $g \approx 10$ is detectable. If this signal belongs to the same state than that from which the $g = 4.1$ signal arises, this state would have a value $S = {}^5/_2$. Nevertheless, a mathematical treatment (*35*, *36*) done on spectra in Figure 1C and which results in a two-dimensional anticorrelation spectrum shows that the resonance at $g = 4.1$ is strictly anticorrelated to the other resonances with g values above 5.

In the model presented above, we still cannot specify whether the photochemistry induces electron transfer within the cluster or a spin state change in the Mn^{III} ion (*21*, *23*) (see the introductory section of the paper). In untreated PSII, the negative value of D for the $S = {}^5/_2$ state of the Mn_4 complex could indicate an elongation of the axial distance between the Mn^{III} ion and the axial ligand(s) (*37*).

The temperature-dependent conversion of the $S = {}^5/_2$ state into the $g = 4.1$ state could indicate a relaxation of the ligand environment around the Mn_4 cluster which consequently would induce a change in the zero-field splitting parameters. In a model in which the $g = 4.1$ signal is assumed to arise from a $S = {}^5/_2$ state, the E/D ratio would be close to 0.3. Nevertheless, we cannot actually rule out a $S = {}^3/_2$ state for the $g = 4.1$ signal.

The dominant feature of the Ca^{2+}-depleted, EGTA-treated, polypeptides-reconstituted PSII sample and Sr^{2+}-reconstituted sample is a large feature with a zero-crossing *g* value close to 9. Assuming a $S = {}^5/_2$ state, the field position of this feature along with those at $g = 4.2$ and the shoulder at $g = 10$ could be readily reproduced using a E/D value 0.21. The large feature arises from the Kramer's doublet corresponding to $\pm{}^1/_2$ states with *g* values 1.9, 9.2, and 1.1. Nevertheless, there are some difficulties to simulate all the features in spectrum b in Figure 3. This could be due to some heterogeneity in the state responsible for the spectrum. In particular, the resonance at $g \approx 4$ could represent a small fraction of centers manifesting the well-known $g = 4$ type signal.

We cannot rule out the possibility that the new EPR signals from the modified enzyme arise from the Mn_4 complex with a spin state > ${}^5/_2$ [see for example some model compounds with a spin state > ${}^5/_2$ (*38*, *39*)]. That said, in untreated PSII, the good quality of the simulated spectrum indicates that the $S = {}^5/_2$ state is the most probable spin state.

In untreated PSII, the yield of the new state is ~40% at 65 K and this substoichiometry may represent some kind of heterogeneity of states which may be in equilibrium at room temperature but which is frozen in at 65 K. The decrease in the yield down to ~10−20% at 10 K may be a further reflection of such a freezing-induced heterogeneity. The formation of a small but significant fraction of $g = 10$ and 6 at 10 K seems to argue against one of the suggestions that we made earlier (*21*): that the state which absorbed the 820 nm light was an excited state rather the $S = {}^1/_2$ ground state.

The new spectra reported above represent the third EPR spectral form arising from the PSII Mn_4 cluster in the S_2 state. The EPR characteristics of this new form (i.e., broad spectra with a small amplitude) may explain some earlier data where the S_2 state was present and yet not detectable as either a $S = {}^1/_2$ multiline signal or a $g = 4.1$ EPR signal [in Cl^--depleted PSII preparation for example (*40*)].

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