THE ORIGIN OF THE MULTILINE AND $g = 4.1$ ELECTRON PARAMAGNETIC RESONANCE SIGNALS FROM THE OXYGEN-EVOLVING SYSTEM OF PHOTOSYSTEM II

ÖRJAN HANSSON, ROLAND AASA, AND TORE VÄNNGÅRD
Department of Biochemistry and Biophysics, Chalmers Institute of Technology, and University of Göteborg, S-412 96 Göteborg, Sweden

ABSTRACT Continuous illumination at 200 K of photosystem (PS) II-enriched membranes generates two electron paramagnetic resonance (EPR) signals that both are connected with the S_2 state: a multiline signal at $g \simeq 2$ and a single line at $g = 4.1$. From measurements at three different X-band frequencies and at 34 GHz, the g tensor of the multiline species was found to be isotropic with $g = 1.982$. It has an excited spin multiplet at ~30 cm^{-1}, inferred from the temperature-dependence of the linewidth. The intensity ratio of the $g = 4.1$ signal to the multiline signal was found to be almost constant from 5 to 23 K. Based on these findings and on spin quantitation of the two signals in samples with and without 4% ethanol, it is concluded that they arise from the ground doublets of paramagnetic species in different PS II centers. It is suggested that the two signals originate from separate PS II electron donors that are in a redox equilibrium with each other in the S_2 state and that the $g = 4.1$ signal arises from monomeric Mn(IV).

INTRODUCTION

Oxidation of water to oxygen in higher plants and algae is catalyzed by photosystem (PS) II. By controlled illumination, it is possible to generate different intermediates in this cyclic reaction. The intermediates, designated S_0, S_1, S_2, and S_3, are characterized by successively higher oxidation states of the redox components of the PS II electron donor side. A majority of the PS II centers reside in the S_1 state in the dark. Oxidation of the S_3 state results in the formation of oxygen and a transition to the S_0 state, from which the process starts over again (reviewed in references 1 and 2).

S_2 is paramagnetic with a multiline electron paramagnetic resonance (EPR) signal at $g \simeq 2$ (3). It is generally agreed upon that there are four manganese ions per PS II, and the signal has been assigned to a bi- or tetranuclear mixed-valence manganese complex (3–8). This is coordinated to oxygen from water, as shown by ^{17}O experiments (9).

For a proper theoretical description of the multiline signal, it is necessary to know the g-anisotropy of the species. Studies on partially oriented chloroplasts (6) or PS II membranes (10) did not reveal any considerable g-anisotropy. This is confirmed here, where measurements at three different X-band frequencies show that $g = 1.97$. Detection of the multiline signal at 34 GHz allowed the more precise determination $g = 1.982 \pm 0.002$.

A second signal comprising a single line at $g = 4.1$ can also be generated by illumination of PS II (11, 12). This signal has been proposed to arise from a rhombic Fe^{3+} (11) or a tetranuclear 3Mn(III)-Mn(IV) (8) complex. Recently it was found that ethanol, ethylene glycol, or glycerol inhibit the generation of the $g = 4.1$ signal (13).

Both the multiline and the $g = 4.1$ signals can be generated by continuous illumination at 200 K (12–14) or 0°C in the presence of 100 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (in dimethyl-sulfoxide) (14) or by flash illumination at 0°C (13), followed by rapid freezing of the samples. The yield of both signals varied with flash number with maxima after one and five flashes (13). These results strongly suggest that both signals are connected with the S_2 state. Other assignments of the $g = 4.1$ signal (11, 12) were based on experiments with ethanol (13). However, in references 11 and 15 it was found that illumination at 140 K only generated the $g = 4.1$ signal, while subsequent thawing to 200 K resulted in an interconversion of this signal to the multiline signal. This points to the possibility of a heterogeneity in the S_2 state.

In the present work, the relation between the multiline and $g = 4.1$ signals was further investigated in an attempt to give an explanation for their appearance. In an oligonuclear mixed-valence manganese complex, one would expect other spin multiplets separated from the EPR observable doublet ($S = 1/2$) with energies determined by the strength of the exchange interactions. Progressive microwave power saturation studies indicated the presence of an excited spin multiplet at ~30 cm^{-1} (6). This is confirmed here from studies of the temperature dependence of the

linewidth of the multiline signal. For a binuclear mixed-valence manganese complex where the electron spins of the ions are antiferromagnetically coupled, the first excited spin multiplet is expected to be a quartet ($S = 3/2$). It is tempting to ask if the $g = 4.1$ signal could originate from such a quartet in the same paramagnetic species that gives rise to the multiline signal. Indeed, an excited quartet at ~400 cm^{-1}, giving rise to a signal at $g = 4$, has been observed in single crystals of a di-μ-oxo-(bipyridine)Mn(III,IV) dimer (16).

However, from a study of the temperature dependence of the multiline and $g = 4.1$ signals and the observation of the multiline signal at 34 GHz, it is concluded here that the signals arise from the ground doublets of different paramagnetic species. It is further concluded, partly based on spin quantitation, that the two species reside in different PS II centers. It is proposed that the two signals originate from separate PS II electron donors that are in a redox equilibrium with each other in the S_2 state and that the $g = 4.1$ signal arises from monomeric Mn(IV).

MATERIALS AND METHODS

Oxygen-evolving (600 μmol O_2/mg chlorophyll[Chl]/h) PS II-enriched membranes (17 mg Chl/ml) were prepared from spinach (17) in a final buffer containing 20 mM MES-NaOH (pH 6.3), 400 mM sucrose, 15 mM NaCl, and 5 mM $MgCl_2$. EPR samples were dark-adapted for 1 h. Where indicated, 4% ethanol was added before freezing the samples at 200 K. Light-induced EPR signals were generated by continuous illumination at 200 K (9) and the samples were subsequently stored at 77 K. In separate experiments it was found that the signals were proportional to the chlorophyll concentration up to 17 mg Chl/ml.

EPR measurements at 9.46 GHz were made with a model ER 200D-SRC spectrometer (Bruker, Karlsruhe, FRG) (9). A model E-231 cavity (Varian, Palo Alto, CA) (operating mode, TE_{102}) was used for studies at somewhat lower microwave frequencies. Insertion of a quartz rod (cross section, 1 × 10 mm^2) through an optical window at the front of the cavity lowered the resonance frequency from 9.25 to the minimum available klystron frequency, 9.08 GHz. Measurements at 34 GHz were made with a spectrometer console (model V-4503; Varian). The output from the V-4560 100 kHz modulation unit was fed via a differential amplifier to the time base ER001 of the Bruker console and to the computer interface ER144C of an Aspect 2000 computer, used for accumulation of spectra. A homebuilt helium cryostat (18) was used. Digital filtering of 34 GHz spectra was done with a convolution difference procedure, involving exponential multiplication of Fourier-transformed spectra. The magnetic field was calibrated with a nuclear magnetic resonance (NMR) magnetometer, and the NMR-frequency was measured with an electronic counter. The same counter was used for measurements of X-band frequencies, while the 34 GHz frequency was determined from the known *g*-value, 2.0036, of α, α'-diphenyl-β-picryl hydrazyl (DPPH).

As a measure of the linewidth of the multiline signal, the ratio $W = 2a/(b + c)$ was calculated, see Fig. 1. This ratio corresponds to the reciprocal of the ratio K defined in reference 19.

RESULTS

Illumination of PS II-enriched membranes at 200 K generates the multiline signal at $g = 2$ and the $g = 4.1$ signal (Fig. 1 *A*), as well as the so-called $g = 1.8$ signal from the $Q_A^-Fe^{2+}$ complex of the acceptor side (20–22), characterized by a peak at $g = 1.84$ and a trough at $g = 1.66$ (Fig. 2 *A*). Addition of 4% ethanol has no significant effect on the yield of the $g = 1.8$ signal (Fig. 2 *B*) but inhibits the formation of the $g = 4.1$ signal (Fig. 1 *B*) and results in an enhanced (Fig. 1 *B*), more easily saturable multiline signal with a reduced intrinsic linewidth (Fig.

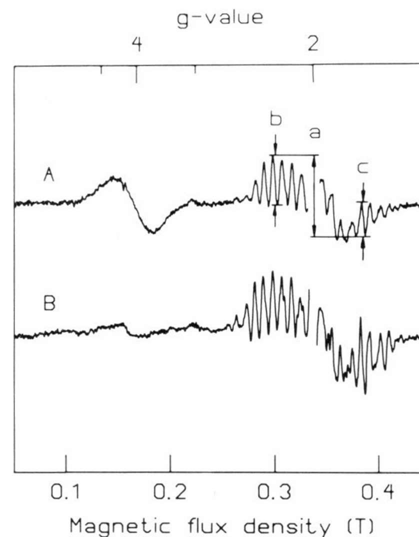


FIGURE 1 Effect of ethanol on the light-induced multiline and $g = 4.1$ EPR signals obtained from a PS II preparation. PS II enriched membranes (17 mg Chl/ml) in EPR tubes were illuminated at 200 K. *A*, sample without ethanol; *B*, a sample to which 4% ethanol had been added before freezing. Spectrometer conditions: microwave frequency, 9.460 GHz; power, 2 mW; modulation amplitude, 2.5 mT; temperature, 12 K. Spectra obtained before the illumination have been subtracted. The amplitudes that were used in the linewidth studies are indicated in *A*.

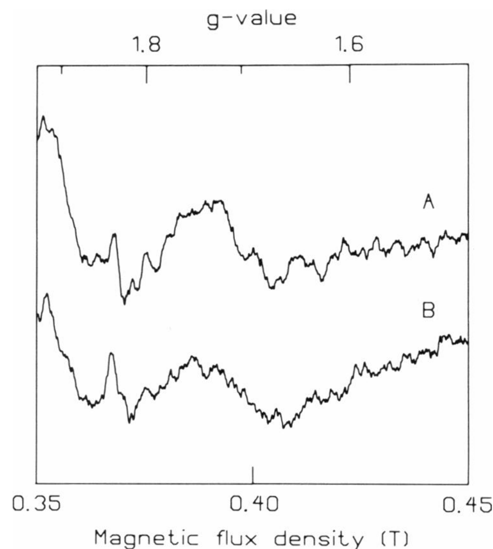


FIGURE 2 EPR signals from the $Q_A^-Fe^{2+}$ complex of the PS II acceptor side induced by continuous illumination at 200 K of PS II-enriched membranes. Spectra were taken from the same samples without (*A*) and with 4% ethanol (*B*), respectively, that were used for Fig. 1. Spectrometer conditions: microwave frequency, 9.46 GHz; power, 80 mW; modulation amplitude, 2.5 mT; temperature, 5 K. the $g = 1.98$ multiline signal is partly saturated under these conditions. Spectra obtained before the illumination have been subtracted.

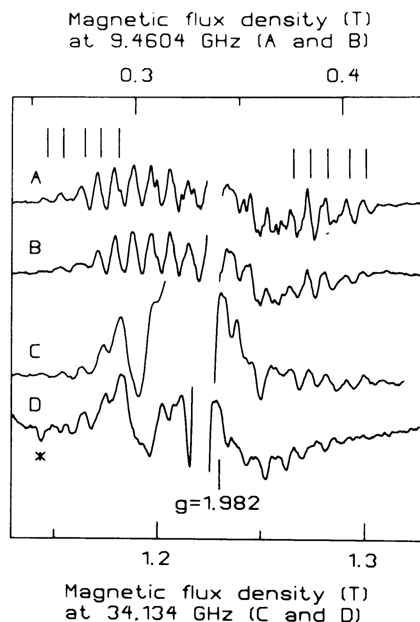


FIGURE 3 Effect of ethanol on the light-induced multiline EPR signal from a PS II preparation measured at 9 and 34 GHz. Samples with (*A* and *D*) or without (*B* and *C*) 4% ethanol were prepared as in Fig. 1. Spectrometer conditions for *A* and *B* (*C* and *D* in parentheses): microwave frequency, 9.4604 (34.134) GHz; power, 20 (10) mW; modulation amplitude, 1.25 (2.1) mT; temperature, 11 (15) K. The spectra in *A* and *B* were slightly saturated under these conditions. Spectra obtained before the illumination have been subtracted in *A* and *B*. In *C* and *D* a convolution difference procedure (see Materials and Methods) was used to subtract a baseline. With this procedure it was not possible to entirely suppress a signal at 1.19 T due to a cavity contamination. The vertical bars in *A* mark the peaks that were studied at three different X-band frequencies. All spectra have been aligned on $g = 1.982$. The feature marked with * was not reproducible.

3, *A* and *B*). These results are consistent with earlier findings (13).

The *g*-Tensor of the Multiline Signal

Measurements at X-Band. To estimate the degree of anisotropy in the multiline signal, measurements were performed at three different X-band frequencies. There was no change in the shape in any of the individual peaks upon changing the frequency, but the position of the peaks changed as shown in Table I. There were no peaks outside the field region of Table I (see Fig. 3 *A*). Second-order hyperfine contributions are virtually constant in this frequency range (estimated with the formulas in reference 4, they change only by ~0.1 mT). Thus, the *g*-values of the individual peaks can be obtained from the slope in a plot of their position versus frequency. All *g*-values are the same within the accuracy of the determination (Table I). The average value obtained, $g = 1.97$, indicates a small, if any, *g*-anisotropy.

Measurements at 34 GHz. A more precise *g*-value can, in principle, be obtained from studies at higher microwave frequencies. Therefore, measurements were performed at 34 GHz, where it was possible to detect the multiline signal both in samples without (Fig. 3 *C*) and with 4% ethanol (Fig. 3 *D*). No peaks could be found outside the region shown in Fig. 3, *C* and *D*. A good correspondence between X-band and 34-GHz spectra from samples without ethanol (taking second-order hyperfine effects into account with the formulas in reference 4) was obtained when they were aligned on $g = 1.982 \pm 0.002$ (Fig. 3, *B* and *C*). Other alignments, obtained by shifting the X-band and 34-GHz spectra an integral number of peak separations (0.01 *g*-value units for each shift), resulted in less good fits.

Addition of 4% ethanol resulted in a small shift in some of the peaks in the 34-GHz spectrum (Fig. 3 *D*).

Linewidth of the $g = 1.98$ Multiline Signal

Measurements were made of the amplitude ratio, *W*, of the $g = 1.98$ multiline signal, as defined in Materials and Methods. This ratio has been used earlier in linewidth studies on similar EPR signals, consisting of a multitude of lines separated by hyperfine splittings comparable to the linewidth (19). This amplitude ratio was found to be directly proportional to the true linewidth (19).

In the temperature interval used, *W* increased with

TABLE I
MAGNETIC FIELD POSITIONS AT THREE DIFFERENT X-BAND FREQUENCIES AND *g*-VALUES OF 10 PEAKS* IN THE MULTILINE EPR SIGNAL

Microwave frequency	Magnetic field									
GHz	*mT*									
9.0754	243.8	251.5	262.1	269.9	278.4	363.2	371.0	379.2	389.9	397.5
9.2468	250.1	257.5	268.2	276.2	284.6	369.1	377.3	385.4	396.2	404.1
9.4592	258.0	265.3	276.1	283.9	292.4	376.7	384.9	392.8	403.9	411.6
g-value‡	1.94	1.98	1.96	1.96	1.97	1.97	1.98	2.01	1.96	1.95

Mean *g*-value ± 2 standard deviations: 1.97 ± 0.04.
*The positions are marked with vertical bars in Fig. 3 *A*. Spectrometer conditions as for Fig. 3 *A*, but modulation amplitude, 2 mT.
‡The *g*-value for each peak was obtained from the slope in a plot of magnetic field position versus frequency.

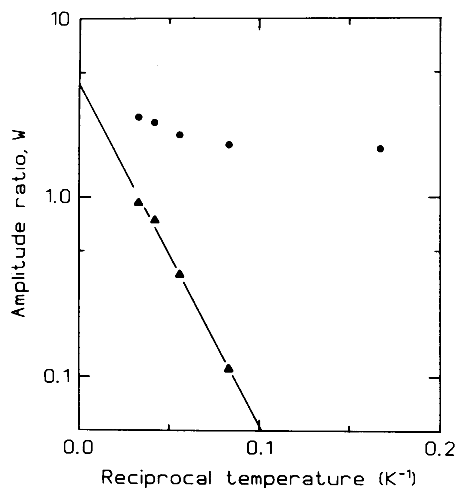


FIGURE 4 Temperature dependence of the linewidth of the $g = 1.98$ multiline signal. Light-minus-dark difference spectra from a sample without ethanol were obtained as in Fig. 1 *A* and corrected for a sloping baseline. The amplitude ratio, $W = 2a/(b + c)$, of the $g = 1.98$ multiline signal, as defined in Materials and Methods (see Fig. 1 *A*), represents the average obtained from up to six different spectra. The temperature dependent part of *W* (*triangles*) was obtained by subtracting the value at 6 K. The line represents the best fit to the data. Its slope, $-\Delta/k$, corresponds to $\Delta = 31\ cm^{-1}$.

temperature. This is shown in Fig. 4 for the sample without ethanol. If it is assumed that *W* is directly proportional to the true linewidth, then one would expect that δW, the temperature dependent part of *W*, should be proportional to the spin-lattice relaxation rate (lifetime broadening) (23). Fitting the data from the sample without ethanol to $\delta W = \text{constant} \cdot \exp(-\Delta/kT)$, which is the temperature dependence predicted for an Orbach relaxation mechanism via an excited spin multiplet at an energy Δ, resulted in $\Delta = 31\ cm^{-1}$ (Fig. 4, *triangles*). A Raman relaxation mechanism, $\delta W = \text{constant} \cdot T^n$, gave an unsatisfactory fit (not shown). A similar value of $|\Delta|$, $30\ cm^{-1}$, was earlier found from a sample containing ethanol (as a solvent for phenyl-*p*-benzoquinone) (6), using a saturation method.

Quantitative Estimates of the $g = 1.98$ Multiline Signal

The absolute concentration of the species giving rise to the $g = 1.98$ multiline signal can be obtained if its double integral is compared with that of a paramagnetic species with a known concentration (24). The accuracy in such determinations is seldom better than ±5%, but the spin concentrations can be of considerable help when judging the significance of different EPR signals. Using 1-mM high-spin metmyoglobin ($D = 9.14\ cm^{-1}$ [25]) as a concentration standard, double integration from 0.24 to 0.44 T of the light-minus-dark difference spectra in Fig. 1 resulted in the concentrations 43 and 28 μM for the samples with and without ethanol, respectively. Assuming 250 Chl per PS II (Chl concentration, 17 mg/ml), these values transform into 0.57 and 0.37 spins per PS II at 12 K, respectively.

Corrections for Overlapping Signals. The values obtained represent overestimates of the true spin concentrations, since there are other light-induced EPR signals in the magnetic field range used in the integrations. However, their contributions can be estimated as follows.

The continuous illumination at 200 K of the samples used here generates ~0.1 spins per PS II of photooxidized cytochrome b_{559}, as estimated from an integration of its $g = 3.0$ peak (26). However, because of microwave power saturation and the integration interval used, only 10% of this will contribute to the double integral of the spectra in Fig. 1.

Superimposed on the $g = 1.98$ multiline signal is also a light-induced signal at $g \sim 1.9$ (11, 12, 15), which is more easily seen at higher temperatures. This signal can also be seen in a sample illuminated at 200 K in which the $g = 1.98$ multiline signal was absent because of treatment with NH_2OH (O. Hansson and L. -E. Andréasson, unpublished results). The signal so obtained (not shown) resembled signals from reduced centers A and B of PS I (27), and it is therefore proposed that the $g = 1.9$ feature is due to a small (3%) PS I contamination, which also must be subtracted from the double integral. The signal is saturated to one-third under the conditions of Fig. 1. Photooxidized P700, the primary electron donor of PS I, will not show up in the integrated difference spectra because of the digitizing procedure (see Fig. 5).

The contribution from the $Q_A^- Fe^{2+}$ complex is more difficult to estimate because of the large anisotropy in its EPR signal (22). This signal can be seen at lower temperatures and higher microwave powers (Fig. 2). The Boltzmann-weighted contribution to the double integral between 0.24 and 0.44 T from the two lowest doublets of the species was estimated from Eq. 23a and Fig. 14 *A* of reference 22 to be 0.2 spins per PS II at 12 K. However, the work in reference 21 indicates, for reasons that are not well understood, that the $g = 1.8$ signal is only developed to 10% of its maximal amplitude under conditions similar to those of the present work. Therefore, its contribution to the double integral of the spectra in Fig. 1 is probably only ~0.02 spins per PS II.

Considering the overlapping signals, the $g = 1.98$ multiline signals will correspond to 0.53 and 0.33 spins per PS II at 12 K in the samples with and without ethanol, respectively.

Corrections for Distribution in Populations. The corrected spin concentrations deviate significantly from that of PS II. However, based on measurements in this laboratory of the oxygen-evolving capacity of chloroplasts, it is estimated that only 75% of the PS II centers are active in the preparation used here. It is further assumed that only 75% of the active PS II centers are in the S_2 state

after the 200 K illumination. With these corrections, the $g = 1.98$ multiline signals at 12 K in the samples with and without ethanol correspond to $0.53/(0.75 \times 0.75) = 0.94$ spins and $0.33/(0.75 \times 0.75) = 0.59$ spins, respectively, per PS II in the S_2 state. Because of the approximations involved, the accuracy in the obtained spin concentrations is estimated to be ±10%.

Temperature Dependence of the $g = 1.98$ Multiline and $g = 4.1$ Signals

Can the $g = 4.1$ signal originate from another spin multiplet, e.g., a quartet, of the same paramagnetic species that gives rise to the $g = 1.98$ multiline signal? If that was the case, then the intensity ratio of the $g = 4.1$ signal to the $g = 1.98$ multiline signal should depend on temperature approximately as $\exp(-\Delta/kT)$, where Δ is the energy splitting of the two multiplets. In Fig. 5 are shown the integrated $g = 1.98$ multiline and $g = 4.1$ signals at three different temperatures. The area under the $g = 4.1$ signal was determined by integration from 0.10 to 0.22 T, while the $g = 1.98$ multiline signal was integrated and corrected for underlying signals as above. The area ratio of the $g = 4.1$ signal to the corrected $g = 1.98$ multiline signal was 0.37 at 23 K, 0.38 at 18 K, 0.35 at 12 K, and 0.32 at 5 K. If the signals stemmed from two spin multiplets of the same paramagnetic species, separated by an energy Δ, then $|\Delta|$ must be $<1\ cm^{-1}$.

Quantitative Estimate of the $g = 4.1$ Signal

From the area ratio of the $g = 4.1$ signal to the $g = 1.98$ multiline signal determined above, it is possible to estimate the spin concentration of the species that gives rise to the $g = 4.1$ signal. The intensity factor g_P^{av} (24) of the $g = 1.98$ multiline signal is simply its *g*-value, while for the $g = 4.1$ signal it is 3.5, if it is assumed that it originates from the ground doublet of a nearly axial $S = 3/2$ species with the effective *g*-values $g_x = 4.2$, $g_y = 4.0$, and $g_z = 2.0$. A simulation (24) with these *g*-values shows that an integration of the $g = 4.1$ signal, taken from 0.10–0.22 T, only includes ~50% of the total intensity. Thus, one arrives at a concentration of 0.13 spins per PS II, or $0.13/(0.75 \times 0.75) = 0.23 \pm 0.03$ spins per PS II in the S_2 state for the $g = 4.1$ signal at 12 K.

DISCUSSION

The Multiline Signal Originates from an Isolated Doublet with the Isotropic *g*-Value 1.98

From the measurements at three different X-band frequencies and at 34 GHz, it is concluded that the *g*-tensor of the multiline signal is isotropic with $g = 1.982 \pm 0.002$. This contrasts the work in reference 8, where an appreciable *g*-anisotropy ($g_x = 1.810$, $g_y = 1.960$, $g_z = 2.274$) was introduced for a simulation. Such an anisotropy would have been observed in the present experiments, particularly at the high- and lowfield wings of the spectrum.

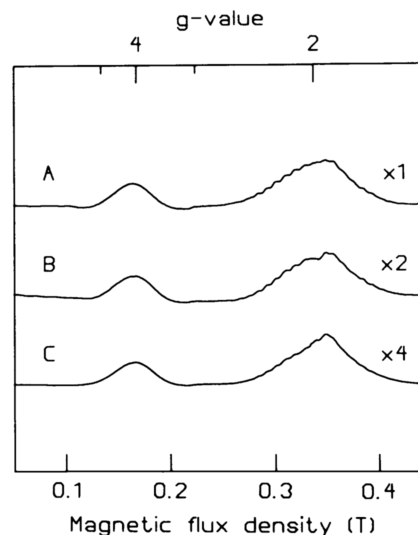


FIGURE 5 Temperature dependence of the light-induced $g = 1.98$ multiline and $g = 4.1$ signals from a PS II preparation. A sample was prepared as in Fig. 1 *A*. Difference (light-minus-dark) spectra at three different temperatures were corrected for a sloping baseline and then integrated once. Spectrometer conditions: microwave frequency, 9.460 GHz; power, 0.05 (*A*), 0.2 (*B*), 20 (*C*) mW; modulation amplitude, 2.5 mT; temperature, 5 (*A*), 12 (*B*), 23 (*C*) K; relative gain, taking microwave power and spectrometer gain into account, as indicated in the figure.

Spectral similarities with low-molecular-weight complexes (28, 29) point to bi- or tetranuclear mixed-valence manganese structures as origins for the signal (3–8, 30). The *g*-value obtained here could be compared with the value $g = 2.003$ estimated for a di-μ-oxo(phenanthroline)Mn(III,IV) dimer (28) and to the values $g_x = g_y = 2.006$, and $g_z = 2.00$ estimated for the Mn(II,III) dimers derived from O_2 oxidation of Mn(II) Schiff-base complexes (29). The deviations are not considered large enough to rule out a binuclear structure as the origin of the $g = 1.98$ multiline signal.

The splitting between the outermost peaks in Table I amounts to 154 mT, and no peaks could be found outside this field region (see Fig. 3). A model involving a tetranuclear manganese complex with both ferro- and antiferromagnetic interactions between the ions as suggested in reference 30 predicts numerous lines outside this range. Since no such lines could be detected in the present work, such a model seems unwarranted. However, a tetranuclear structure with antiferromagnetic interactions between all ions cannot be ruled out since the resulting hyperfine structure could be the same as that from a binuclear complex (30).

Since the $g = 1.98$ multiline signal can be observed at 34 GHz (microwave quantum, $1.1\ cm^{-1}$), the signal must arise from a doublet separated from other spin multiplets by more than a few cm^{-1} at zero magnetic field. Otherwise

the signal would have been much distorted (probably beyond detection) at 34 GHz.

The ethanol-induced shift of some of the lines observed at 34 GHz is possibly due to a small g-anisotropy. However, this is probably too small to be observed at X-band and will not have to be included in simulations of X-band spectra. The small anisotropy observed at X-band in reference 10 is probably due to hyperfine anisotropy.

The $g = 1.98$ Multiline Signal Relaxes via an Excited Spin Multiplet at $\sim 30\ cm^{-1}$

Previous microwave power saturation studies (6, 7) and the present linewidth studies indicate the presence of an excited spin multiplet at $\sim 30\ cm^{-1}$ in the $g = 1.98$ multiline species. Thus, in agreement with the earlier conclusion (6), the EPR signal probably relaxes through an Orbach relaxation mechanism via the excited spin multiplet.

The lifetime broadening observed here indicates that there is a significant Lorentzian contribution to the linewidth. Hyperfine anisotropy will also contribute to the wings of the individual lines, giving them a Lorentzian character. This leads to a considerable overlap between individual lines so that the spectrum apparently looks like a superposition of a broad spectrum and a spectrum with many narrow lines (see Fig. 1 and reference 19). This explains why previous simulations of the $g = 1.98$ multiline signal, based on the assumption of Gaussian lineshapes, could not account for the broad feature (3, 6) and why an earlier estimate of the spin concentration of the $g = 1.98$ multiline signal, based on the same assumption, gave a low value (6).

The ethanol-induced linewidth decrease in the $g = 1.98$ multiline signal reported here and in reference 13 is possibly due to a decrease in the transverse relaxation rate, T_2^{-1}, resulting in a signal that also is more easy to saturate (13). The reduced linewidth can be of advantage in ligand-substitution experiments as exemplified by the study in reference 9, where it was possible to detect an ^{17}O-induced broadening of the $g = 1.98$ multiline signal in the presence of ethanol (used as a solvent for the electron acceptor phenyl-p-benzoquinone).

The $g = 1.98$ Multiline and $g = 4.1$ Signals Originate from the Ground Doublets of Different Paramagnetic Species

The temperature study of the $g = 1.98$ multiline and $g = 4.1$ signals was made to investigate the possibility that the two signals arise from two spin multiplets of the same paramagnetic species. If this was the case, the weak temperature dependence in their area ratio alone suggests that the energy splitting of the multiplets must be $< 1\ cm^{-1}$. However, this possibility is not compatible with the appearance of the $g = 1.98$ multiline signal at 34 GHz (see above). It must therefore be concluded that the $g = 1.98$ multiline and $g = 4.1$ signals originate from different paramagnetic species.

The weak temperature dependence in the ratio of the signals indicates that they arise either from ground spin multiplets or from excited spin multiplets that happen to be at the same energy above their respective ground states. The latter possibility is considered unlikely and is also at variance with the estimated spin concentration of the $g = 1.98$ multiline signal. If this signal was due to an excited doublet a few reciprocal centimeters above an EPR-invisible quartet ground spin multiplet (7), only one-third or less of the $g = 1.98$ species would be populated in the EPR-visible doublet, resulting in an effective spin concentration much lower than observed.

The conclusion that the $g = 1.98$ multiline and $g = 4.1$ signals both arise from ground spin multiplets contrasts that reached in reference 7, where deviations from Curie-law behavior below 8 K were taken as evidence that the $g = 1.98$ multiline signal arises from an excited doublet. One possible cause for this discrepancy could be a difference in sample preparations. To investigate this, separate experiments were performed on samples prepared according to the procedures used in reference 7. The amplitude of the $g = 1.98$ multiline signal was studied between 2.3 and 10 K. No deviation from the Curie law could be observed (R. Aasa, L. -E. Andréasson, and T. Vänngård, unpublished data).

The $g = 1.98$ Multiline and $g = 4.1$ Signals Originate from Different PS II Centers

Can the two paramagnetic species reside in the same PS II unit? This would provide an attractive explanation for the fact that the $g = 1.98$ multiline signal is more difficult to power saturate in the presence of the $g = 4.1$ signal than in its absence. However, since both signals are produced with the experimental protocol used here and with a single flash (13) they cannot arise from oxidation of two different species in the same center, since only one electron is transferred from the donor to the acceptor side.

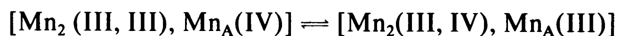
One could think of another possibility, namely that the oxidation of the $g = 1.98$ multiline species induces a conformational change of the $g = 4.1$ species, changing its magnetic properties so that it becomes EPR-visible, without changing its oxidation state. However, with this model it is hard to explain why the spin concentration of the $g = 1.98$ multiline species increases when the formation of the $g = 4.1$ signal is inhibited by ethanol. Indeed, the spin concentration of the $g = 4.1$ species balances the ethanol-induced increase in the $g = 1.98$ multiline signal if appropriate Boltzmann factors are taken into account. The spin concentrations were obtained at 12 K. Assuming an excited quartet at $30\ cm^{-1}$ in the $g = 1.98$ multiline species and that the $g = 4.1$ signal arises from the ground doublet of an $S = 3/2$ species with a zero-field splitting of $1\ cm^{-1}$, the actual concentrations will be ~ 1.0 and 0.6 per PS II in

the S_2 state for the $g = 1.98$ multiline species in the samples with and without ethanol, respectively, and ~0.4 per PS II in the S_2 state for the $g = 4.1$ species in the sample without ethanol. Thus, one arrives at the conclusion that the $g = 4.1$ and $g = 1.98$ multiline signals originate from different PS II centers.

A Model for the Origin of the $g = 1.98$ Multiline and $g = 4.1$ Signals

Two possibilities can be considered. The $g = 1.98$ multiline and $g = 4.1$ signals could arise either from the same redox component existing in two different conformations or from two different redox components in a redox equilibrium with each other. The first possibility was suggested in references 8, 13, 15, and in reference 8 it was proposed that both signals originate from the same tetranuclear 3 Mn(III)-Mn(IV) complex in two different conformations characterized by different strengths of the exchange interactions. Since the present work shows that both signals arise from ground spin multiplets, this model would require large changes in the exchange interaction to explain the interconversion between two signals, which is observed on increasing the temperature from 140 to 200 K (11, 15). This seems less likely, and as an alternative, it is suggested here that the two EPR signals arise from different redox components of the PS II donor side. A plausible model could be that the $g = 1.98$ multiline signal originates from a binuclear Mn_2(III,IV) complex, while the $g = 4.1$ signal stems from an almost axial Mn_A(IV) species. Indeed, it is known that low-molecular-weight complexes of Mn(IV) can give rise to EPR signals similar to the $g = 4.1$ signal (31, 32).

Mn_2 and Mn_A are suggested to be in a redox equilibrium with each other in the S_2 state:



$$[Mn_2\,(III, III), Mn_A(IV)] \rightleftharpoons [Mn_2(III, IV), Mn_A(III)]$$

The change in relative amounts of the signals observed upon addition of ethanol, ethylene glycol, and glycerol (13) is proposed to be due to changes in the reduction potentials of the components such that this equilibrium is driven to the right. At 140 K the electron transfer from Mn_2 to Mn_A is inhibited, resulting only in photooxidation of Mn_A, while a temperature increase to 200 K would release an electron from Mn_2, thus explaining the observed interconversion between the two signals (11, 15).

We would like to thank B. Källebring for computer programming, J. -L. Zimmermann for sending a preprint of his work (13) and L. -E. Andréasson, A. W. Rutherford, and T. Wydrzynski for stimulating discussions.

This work was supported by the Swedish Natural Science Research Council.

Received for publication 13 August 1986 and in final form 5 January 1987.

REFERENCES

1. Renger, G., and R. Govindjee. 1985. The mechanism of photosynthetic water oxidation. *Photosynth. Res.* 6:33–35.
2. Dismukes, G. C. 1986. The metal centers of the photosynthetic oxygen-evolving complex. *Photochem. Photobiol.* 43:99–115.
3. Dismukes, G. C., and Y. Siderer. 1981. Intermediates of a polynuclear manganese center involved in photosynthetic oxidation of water. *Proc. Natl. Acad. Sci. USA.* 78:274–278.
4. Hansson, Ö., and L. -E. Andréasson. 1982. EPR-detectable magnetically interacting manganese ions in the photosynthetic oxygen-evolving system after continuous illumination. *Biochim. Biophys. Acta.* 679:261–268.
5. Andréasson, L. -E., Ö. Hansson, and T. Vänngård. 1983. On the role of manganese in photosynthetic oxygen evolution. *Chem. Scr.* 21:71–74.
6. Hansson, Ö., L. -E. Andréasson, and T. Vänngård. 1984. Studies on the multiline EPR signal associated with state S_2 of the oxygen-evolving system. *In* Advances in Photosynthesis Research. Vol. I. C. Sybesma, editor. Martinus Nijhoff/Dr W. Junk Publishers, The Hague. 307–310.
7. de Paula, J. C., and G. W. Brudvig. 1985. Magnetic properties of manganese in the photosynthetic O_2-evolving complex. *J. Am. Chem. Soc.* 107:2643–2648.
8. de Paula, J. C., W. F. Beck, and G. W. Brudvig. 1986. Magnetic properties of manganese in the photosynthetic O_2-evolving complex. 2. Evidence for a manganese tetramer. *J. Am. Chem. Soc.* 108:4002–4009.
9. Hansson, Ö., L. -E. Andréasson, and T. Vänngård. 1986. Oxygen from water is coordinated to manganese in the S_2 state of photosystem II. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* 195:151–154.
10. Rutherford, A. W. 1985. Orientation of EPR signals arising from components in photosystem II membranes. *Biochim. Biophys. Acta.* 807:189–201.
11. Casey, J. L., and K. Sauer. 1984. EPR detection of a cryogenically photogenerated intermediate in photosynthetic oxygen evolution. *Biochim. Biophys. Acta.* 767:21–28.
12. Zimmermann, J. -L., and A. W. Rutherford. 1984. EPR studies of the oxygen-evolving enzyme of photosystem II. *Biochim. Biophys. Acta.* 767:160–167.
13. Zimmermann, J. -L., and A. W. Rutherford. 1986. EPR properties of the S_2 state of the oxygen-evolving complex of photosystem II. *Biochemistry.* 25:4609–4615.
14. Andréasson, L. -E., and Ö. Hansson. 1987. EPR studies of the oxygen-evolving system. The interaction with amines. *In* Progress in Photosynthesis Research. Vol. 1. J. Biggins, editor. Martinus Nijhoff Publishers, Dordrecht. 503–510.
15. de Paula, J. C., J. B. Innes, and G. W. Brudvig. 1985. Electron transfer in photosystem II at cryogenic temperatures. *Biochemistry.* 24:8114–8120.
16. Inoue, M. 1978. ESR studies of an oxo-bridged manganese(III, IV) complex of 2,2′-bipyridine. *Bull. Chem. Soc. Jpn.* 51:1400–1403.
17. Franzén, L. -G., Ö. Hansson, and L. -E. Andréasson. 1985. The roles of the extrinsic subunits in photosystem II as revealed by EPR. *Biochim. Biophys. Acta.* 808:171–179.
18. Albracht, S. P. J. 1974. A low-cost cooling device for EPR measurements at 35 GHz down to 4.8°K. *J. Magn. Res.* 13:299–303.
19. Hyde, J. S., and W. K. Subczynski. 1984. Simulation of ESR spectra of the oxygen-sensitive spin-label probe CTPO. *J. Magn. Res.* 56:125–130.
20. Nugent, J. H. A., B. A. Diner, and M. C. W. Evans. 1981. Direct detection of the electron acceptor of photosystem II. Evidence that Q is an iron-quinone complex. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* 124:241–244.
21. Vermaas, W. F. J., and A. W. Rutherford. 1984. EPR measurements on the effects of bicarbonate and triazine resistance on the

acceptor side of photosystem II. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* 175:243–248.

22. Butler, W. F., R. Calvo, D. R. Fredkin, R. A. Isaacson, M. Y. Okamura, and G. Feher. 1984. The electronic structure of Fe^{2+} in reaction centers from *Rhodopseudomonas sphaeroides*. III. EPR measurements of the reduced acceptor complex. *Biophys. J.* 45:947–973.
23. Beardwood, P., J. F. Gibson, P. Bertrand, and J. -P. Gayda. 1983. Temperature dependence of the electronic spin-lattice relaxation time in a 2-iron-2-sulphur model complex. *Biochim. Biophys. Acta.* 742:426–433.
24. Aasa, R., and T. Vänngård. 1975. EPR signal intensity and powder shapes: a reexamination. *J. Magn. Res.* 19:308:315.
25. Scholes, C. P., R. A. Isaacson, and G. Feher. 1971. Determination of the zero-field splitting of Fe^{3+} in heme proteins from the temperature dependence of the spin-lattice relaxation rate. *Biochim. Biophys. Acta.* 244:206–210.
26. Bergström, J., and T. Vänngård. 1982. EPR signals and orientation of cytochromes in the spinach chloroplast thylakoid membrane. *Biochim. Biophys. Acta.* 682:452–456.
27. Chamorovsky, S. K., and R. Cammack. 1982. Effect of temperature on the photoreduction of centres A and B in photosystem I, and the kinetics of recombination. *Biochim. Biophys. Acta.* 679:146–155.
28. Cooper, S. R., G. C. Dismukes, M. P. Klein, and M. Calvin. 1978. Mixed valence interactions in Di-μ-oxo bridged manganese complexes. Electron paramagnetic resonance and magnetic susceptibility studies. *J. Am. Chem. Soc.* 100:7248–7252.
29. Mabad, B., J. -P. Tuchagues, Y. T. Hwang, and D. N. Hendrickson. 1985. Mixed-valence $Mn^{II}Mn^{III}$ complexes: models for the manganese site in the photosynthetic electron-transport chain. *J. Am. Chem. Soc.* 107:2801–2802.
30. Dismukes, G. C., K. Ferris, and P. Watnick. 1982. EPR spectroscopic evidence for a tetranuclear manganese cluster as the site for photosynthetic oxygen evolution. *Photobiochem. Photobiophys.* 3:243–256.
31. Richens, D. T., and D. T. Sawyer. 1979. Bis(tetramethylammonium)tris(sorbitolato)manganate(IV), an EPR-active monomeric complex of manganese(IV). *J. Am. Chem. Soc.* 101:3681–3683.
32. Magers, K. D., C. G. Smith, and D. T. Sawyer. 1980. Electrochemical and spectroscopic studies of Tris(3,5-di-*tert*-butylcatecholato)manganese(IV) and its dioxygen adduct. *Inorg. Chem.* 19:492–496.