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EPR-DETECTABLE MAGNETICALLY INTERACTING MANGANESE IONS IN THE PHOTOSYNTHETIC OXYGEN-EVOLVING SYSTEM AFTER CONTINUOUS ILLUMINATION

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Freezing of spinach or barley chloroplasts during continuous illumination results in the trapping of a paramagnetic state or a mixture of such states characterized by a multiline EPR spectrum. Added Photosystem II electron acceptor enhances the signal intensity considerably. Treatments which abolish the ability of the chloroplasts to evolve oxygen, by extraction of the bound manganese, prevent the formation of the paramagnetic species. Restoration of Photosystem II electron transport in inhibited chloroplasts with an artificial electron donor (1,5-diphenylcarbazide) does not restore the multiline EPR spectrum. The presence of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) results in a modified signal which may represent a second paramagnetic state. The paramagnetic forms appear to originate on the donor side in Photosystem II and are dependent on a functional oxygen-evolving site and bound, intact manganese. It is suggested that magnetically interacting manganese ions in the oxygen-evolving site may be responsible for the EPR signals. This suggestion is supported by calculations.

Introduction

Although a central role of manganese in the photosynthetic electron transport is generally acknowledged [1], the details of its participation are in most respects obscure. The many possible oxidation states of the manganese ion have suggested a role in the charge accumulation on the donor side of Photosystem II which is associated with the release of oxygen [2]. The dependence of the relaxation of water protons on the number of light flashes given to chloroplasts was claimed as evidence of changes in the oxidation state of photosynthetic manganese [3–5]. These studies have, however, met with some criticism [6]. Support for valence state changes has also been obtained from estimations of the amount of divalent manganese released after heat-treatment of flash-illuminated chloroplasts [7].

Recently, however, Dismukes and Siderer [8] showed that a complex EPR signal, dependent on the number of flashes, could be obtained after freeze-trapping of flash-illuminated chloroplasts. The EPR spectrum observed was in many aspects similar to that obtained from a synthetic Mn(III)/Mn(IV) dimer [9]. The intermediate was suggested to originate from one of the activated states (S_2) of the oxygen-evolving system.

The possibility that one of the S-states could give rise to a paramagnetic, EPR-detectable state prompted us to use a somewhat different approach when generating the signal than that used by Dismukes and Siderer. Continuous illumination of chloroplasts in the presence of the native electron donor (H_2O) and a sufficient concentration of acceptor should result in a steady-state distribution among the possible

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Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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S-states. Provided that the conditions are correctly chosen, the concentration of paramagnetic states (e.g., S_2) after freeze-trapping might be high enough to allow detection by EPR spectroscopy. Using this approach we have observed a strong multiline EPR signal, similar to that reported by Dismukes and Siderer. The signal can be considerably enhanced by the addition of the Photosystem II electron acceptor phenyl-*p*-benzoquinone (PPBQ). The signal is completely abolished by treatments which result in the release of bound manganese and inhibition of oxygen evolution. The signal cannot be generated in manganese-extracted chloroplasts where Photosystem II electron transport has been partially restored by the addition of the artificial electron donor 1,5-diphenylcarbazide. Thus, a functional oxygen-evolving site appears to be a prerequisite for the generation of the multiline EPR signal. In the presence of DCMU a modified signal is observed, possibly related to a second paramagnetic state.

The convenience by which the signals can be produced with the method described in the present communication, should allow detailed investigations of the responsible states and aid in the understanding of the mechanism of oxygen evolution.

Materials and Methods

Broken chloroplasts were prepared in the following way. Fresh leaves, harvested from spinach or young barley plants grown in a cultivator chamber, were ground in a Waring blender for 10 s in a medium consisting of 350 mM sucrose, 50 mM Tricine-KOH, pH 7.5, 10 mM NaCl and 0.5 mM EDTA. The homogenate was strained through four layers of 20-mesh nylon cloth and centrifuged at 3000 × *g* for 5 min. The pellet was then suspended in a dithizon-extracted Hepes buffer (50 mM Hepes-KOH (pH 7.5)/10 mM NaCl), homogenized in a Potter-Elvehjem homogenizer and centrifuged at 5000 × *g* for 3 min. After suspending the pellet in the Hepes buffer, the chloroplast fragments were again centrifuged at 5000 × *g* for 3 min. The pellet was finally suspended in the Hepes buffer to a chlorophyll concentration of 4–9 mg/ml as determined by the method of Arnon [10]. Dithizon-extracted EDTA was added to a concentration of 3 mM and the chloroplast fragments were kept on ice in the dark for 30 min. Photosystem II activity and manganese content, measured as described earlier [11], were over 100 μmol O_2/mg Chl per h and 1 mol Mn/70 mol Chl, respectively.

Chloroplast samples in EPR tubes (i.d. 3 mm) were frozen in liquid nitrogen, either in the dark (controls) or well illuminated with white light from a 250 W tungsten source. The light was passed through 10 cm water and a Calflex B_1K_1 infrared-reflecting filter to give a final irradiance of 1000 W/m^2. Samples were prepared both with and without the addition of the Photosystem II electron acceptor PPBQ, dissolved in methanol [12].

Broken chloroplasts in EPR tubes were subjected to different treatments before freezing. Inhibition of the oxygen-evolving system was accomplished by incubation with 0.8 M Tris-H_2SO_4 (pH 8.0) (dithizon-extracted) for 30 min at 0°C, with 4 mM NH_2OH for 10 min at 0°C or heating to 55°C for 3 min. Restoration of Photosystem II electron transport in chloroplasts, heat-treated as above, was obtained by the addition of the Photosystem II electron donor diphenylcarbazide [13]. PPBQ was then added to the samples which were finally frozen in the same way as the untreated chloroplasts.

In order to limit the number of electrons extracted from the donating side of Photosystem II, the chloroplast fragments were incubated with 75 μM DCMU (which blocks the reduction of plastoquinone) for 5 min before freezing in the presence or absence of PPBQ.

All additions and further handling of the chloroplasts were made in very weak green light. The samples were stored in the dark in liquid nitrogen.

EPR measurements were made in the dark at 9.25 GHz with a Varian E9 spectrometer equipped with an Oxford Instruments ESR-9 helium-flow cryostat. Spectra were recorded at 10 K with a microwave power of 126 mW, a modulation amplitude of 3.2 mT and a constant amplifier gain (unless otherwise stated in the figure legends).

The spectra presented are the results of a total of six scans (three scans in each field direction). The averaging was made with the spectrometer connected on-line to a Nova 3 minicomputer (Data General Corp.), which was also used for baseline adjustments and for obtaining difference spectra. Before presentation on a Hewlett-Packard 7004 A *X-Y* recorder, the spectra were smoothed with a procedure using the average of four succesive data points (each data point corresponding to 0.1 mT).

Results

Broken, dark-adapted spinach chloroplasts, frozen in liquid nitrogen under continuous illumination, develop a weak multiline EPR signal centered around g 2 (Fig. 1a). To examine how the limited availability of the natural electron acceptor in the chloroplasts affects the concentration of the species responsible for the EPR signal, the Photosystem II electron acceptor PPBQ was added in the dark immediately before illumination. This leads to a considerable enhancement of the signal intensity (Fig. 1d). The signal appears to be identical to that observed without the artificial acceptor. The shape of the signal, i.e., the number and positions of the lines, and the conditions for its generation are not dependent on the source of the chloroplasts, whether spinach or barley. Exclusion of the EDTA used to suppress the EPR signal from hexaquo-Mn^{2+} does not affect the properties of the multiline signal.

The multiline EPR signal is conveniently observed

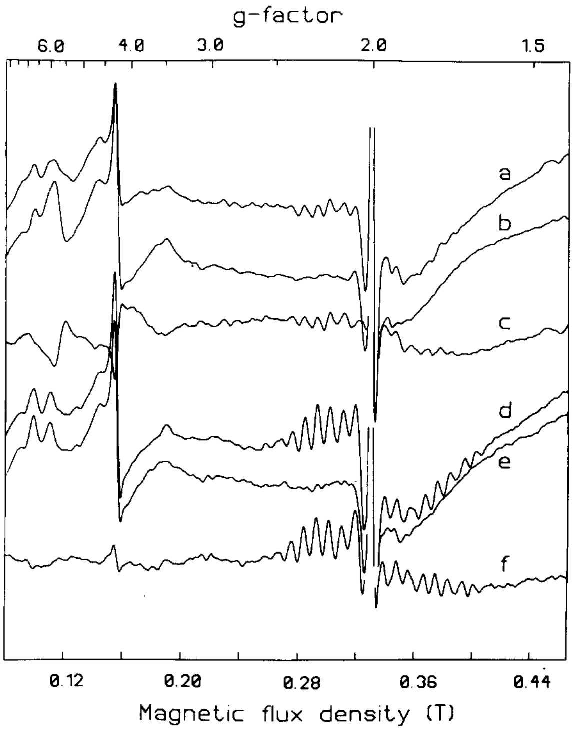

Fig. 1. EPR spectra of dark-adapted and illuminated, broken spinach chloroplasts, recorded at high microwave power and low temperature at 9.25 GHz. The chloroplast samples were prepared as described in Materials and Methods with a chlorophyll concentration of 9 mg/ml in 50 mM Hepes/10 mM NaCl (pH 7.5). Spectrum *a*, chloroplasts frozen under continuous illumination; *b*, frozen in the dark (control); *c*, difference *a minus b*; *d* and *e*, as in *a* and *b*, respectively, but with 4 mM PPBQ; *f*, difference *d minus e*. The conditions for the EPR measurements were: microwave power, 126 mW; modulation amplitude, 3.2 mT; scan rate, 6 mT/s; time constant, 0.1 s; temperature 10 K. Sampling and averaging of spectra as outlined in Materials and Methods.

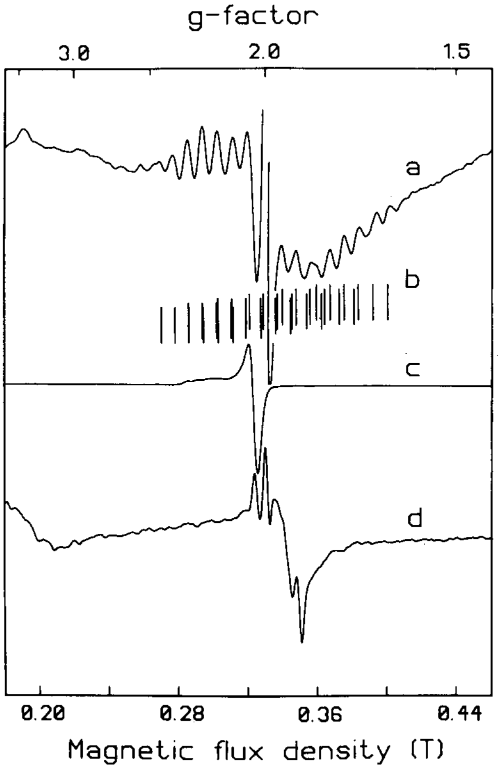

Fig. 2. Contributions from plastocyanin and iron-sulfur centers to the light-induced multiline EPR spectrum from chloroplasts. Spectrum *a*, the same as in Fig. 1*d*; *b*, stick spectrum, calculated assuming an antiferromagnetically coupled Mn(III)/Mn(IV) pair as described in Discussion. Lines with different m_{I1} have been vertically displaced; *c*, purified plastocyanin (50 μM), spectrum recorded under the same conditions as spectrum *a*. The intensity was adjusted to correspond approximately to that of the plastocyanin component in *a* (gain 0.04 relative to *a*); *d*, chloroplasts with the same concentration as in *a* but mixed with 50 mM sodium dithionite and frozen during illumination to reduce the iron-sulfur centers A and B (gain 0.5relative to *a*) Other experimental conditions as in Fig. 1.

only at low temperatures (below 15 K). Interference from plastocyanin, especially at the low-field side of g 2, and from iron-sulfur centers has been largely avoided by using a high power level at the expense of a slightly saturated multiline signal.

The contribution of oxidized plastocyanin to the EPR spectra containing the multiline signal was examined by comparison with a spectrum of purified plastocyanin recorded under the same conditions. Only in a very narrow region around g 2 is there a significant interference from plastocyanin (Fig. 2c). Contributions from reduced Photosystem I iron-sulfur centers may interfere somewhat on the high-field side of g 2 (Fig. 2d). The complete reduction of the sample in Fig. 2d results in a signal intensity much larger than in the partially photoreduced sample in Fig. 2a. Spectrum 2d is shown only to demonstrate the positions of the iron-sulfur signals.

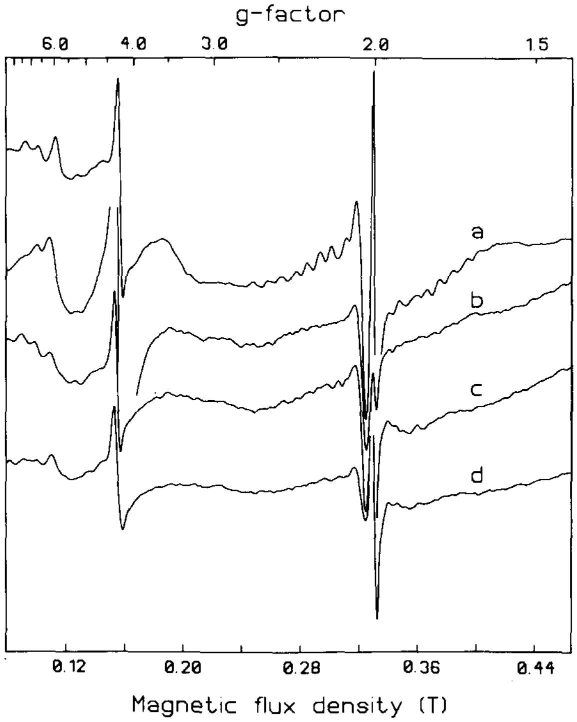

Fig. 3. Effects of inhibition of oxygen evolution by manganese extraction on the light-induced multiline EPR signal in barley chloroplasts. Broken barley chloroplasts, 4 mg chlorophyll/ml, in 50 mM Hepes/10 mM NaCl (pH 7.5) and 4 mM PPBQ were treated in the dark as described in Materials and Methods and frozen under continuous illumination: Spectrum *a*, untreated; *b*, heat treated; *c*, incubated with NH_2OH; *d*, incubated with Tris-H_2SO_4. The conditions for the EPR measurements were as in Fig. 1.

The emergence of the EPR signal is completely inhibited by treatments which destroy the oxygen evolution capability through the release of manganese, i.e., addition of weakly alkaline Tris or hydroxylamine, or by gentle heating (Fig. 3). Restoration of the electron flow in Photosystem II, with the artificial electron donor diphenylcarbazide, in chloroplasts where the water-splitting activity has been specifically eliminated by heat treatment, does not generate the EPR signal (Fig. 4).

The conditions necessary for the formation of the species responsible for the multiline signal was investigated by addition of DCMU in order to block the reduction of the plastoquinone pool. In the presence of DCMU, illumination may lead to an enrichment of one of the S-states. This state should correspond to the number of electrons transferred from the donor to the acceptor side in Photosystem II. DCMU does not prevent the emergence of the EPR signal (Fig. 5c). On the contrary, in experiments without artificial electron acceptor, the presence of DCMU leads to a signal yield significantly higher than that in its absence (Figs. 5a, c). The multiline signal

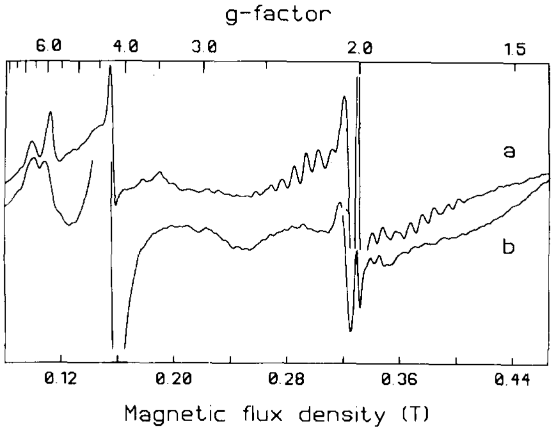

Fig. 4. Effect on the EPR spectrum after partial restoration of Photosystem II electron transport in heat-treated chloroplasts. Spinach chloroplasts, chlorphyll concentration 5 mg/ml, in 50 mM Hepes/10 mM NaCl (pH7.5) were treated as follows: Spectrum *a*, mixed with 4 mM PPBQ and frozen under illumination (control); *b*, warmed at 55°C for 3 min in the dark, mixed with 4 mM PPBQ and 4 mM diphenylcarbazide and frozen under illumination. Conditions for the EPR measurements as in Fig. 1.

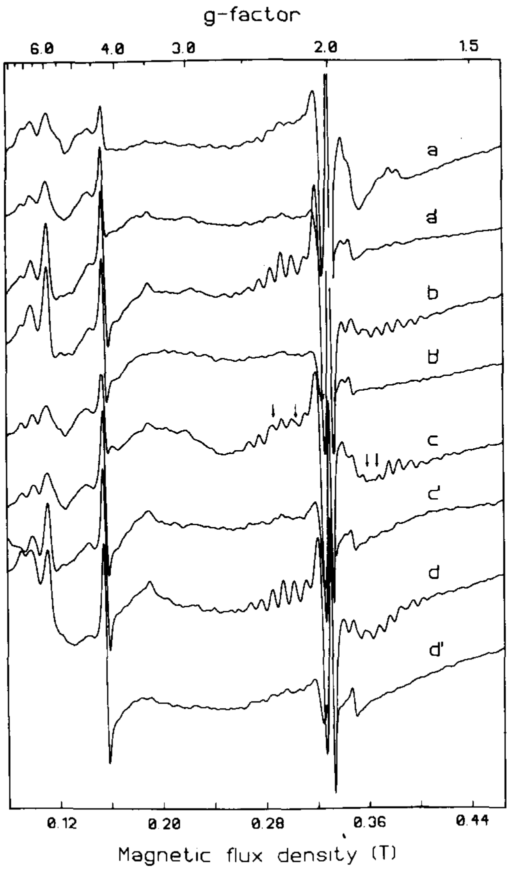

Fig. 5. Effects of DCMU on the light-induced multiline EPR signal in chloroplasts. Spinach chloroplasts, chlorophyll concentration 5 mg/ml, in 50 mM Hepes/10 mM NaCl (pH 7.5), were frozen in continuous light after the following additions: Spectrum *a*, no addition; *b*, 4 mM PPBQ; *c*, 75 μM DCMU; *d*, 4 mM PPBQ and 75 μM DCMU. The samples *a'*–*d'* were treated as *a*–*d*, respectively, but were frozen in the dark as controls. The conditions for the EPR measurements were the same as in Fig. 1.

produced in the presence of DCMU without the artificial acceptor PPBQ (Fig. 5c) differs in some details from that seen with only PPBQ added (Fig. 5b). With PPBQ and DCMU simultaneously present (Fig. 5d) the signal has the same shape and intensity as observed in samples with PPBQ as the only addition. In other words, the effect of DCMU seems to be abolished when PPBQ is present.

EPR spectra were also recorded at lower modulation amplitude in an attempt to improve the resolution (at the expense of the signal to noise ratio). There was no significant further resolution at a modulation amplitude of 0.8 mT (not shown).

In the wide-field spectra several interesting features are noticeable. Over the range *g* 6 to *g* 7 at least two prominent resonances can be detected. These are usually slightly enhanced in samples with added electron acceptor but do not seem to be dependent on illumination to any larger degree (see, for example, Figs. 1 and 5). They do appear, on the other hand, to be perturbed by treatments which affect the oxygen-evolving system (Fig. 3).

Heat treatment of the chloroplasts induces a strongly enhanced signal at *g* 4.3 which is not much further affected by illumination (Figs. 3b, 4b). The possible significance of this signal will be discussed below.

The weak lines barely detectable at *g* 3.5 and *g* 3 (see, for example, Fig. 5b) originate from the oxidized forms of cytochromes *f* and *b*-559, respectively. These signals are strongly saturated under the conditions chosen.

No orientation-dependent signal similar to those observed by Slabas and Evans [14] was detected.

Discussion

The steady-state concentrations of the S-states in continuous light as defined by Kok et al. [2], have been measured [15,16] by monitoring the oxygen evolved by a flash sequence after preillumination by continuous light. It was found that the concentrations of the states (S_0 to S_3) are very similar. This implies that continuous illumination of chloroplasts generates enough of the activated states (S_2 and S_3) to allow detection by a suitable technique. The experiments by Dismukes and Siderer [8] have shown that at least one such state, identified as S_2, is paramagnetic and detectable by EPR spectroscopy. The EPR spectrum of chloroplasts frozen in continuous light (Figs. 1a, d) is similar to that observed by Dismukes and Siderer after a single flash and may be assigned to the same paramagnetic species. Likewise, the origin of the signal produced in continuous light appears to be on the donor side of Photosystem II, more precisely in the oxygen-evolving system, since it is completely eliminated in chloroplasts which have been treated to release the labile manganese [17] and inhibit oxygen evolution. This interpretation is strengthened by the observation that reconsititution of the electron transport by circumventing the water-splitting site with an artificial electron donor, diphenylcarbazide, after heat treatment to release the manganese, fails to produce the signal.

Exhaustive photoreduction of the native electron acceptor, plastoquinone, in the absence of added acceptor, can be assumed to lead to a gradual halt in the Photosystem II electron flow. The concentrations of the various S-states, thereafter, should depend on the extent of excitation and back reactions in Photosystem II. The distribution is expected to be influenced by the presence of added electron acceptor, since the rates of relaxation of the higher states (S_2 and S_3) are known to depend on several factors, among them the redox state of the plastoquinone pool (reviewed in Ref. 18). It is, therefore, not surprising that addition of PPBQ has an effect on the signal strength, since the pool is kept oxidized and the electron flow maintained until the freezing procedure quenches the overall reaction. It is, however, uncertain whether the signal actually found in the samples truly represent the steady-state concentration at room temperature, since the lowering of the temperature upon freezing may shift the steady-state levels of the various S-states.

DCMU is known to inhibit the reoxidation of the primary acceptor, Q, by secondary acceptors and to prevent the formation of the higher S-states (S_3 and S_4) [18]. In the presence of DCMU, therefore, one should expect to see the formation of state S_2 only. Moreover, a relatively strong contribution from this state is expected, since the state S_1 is dominating in the dark [2] and because equalization of the S-states, which occurs in the normal steady state, is inhibited by DCMU. Without added electron acceptor an appreciable enhancement of the EPR intensity is observed with DCMU which indicates that an enrichment of a paramagnetic species takes place which may be tentatively identified as S_2. This signal (Fig. 5c) is significantly different from that observed in unhibited chloroplasts (Fig. 5b). Interference from other paramagnetic compounds, mainly plastocyanin and iron-sulfur centers, does not account for the difference (Fig. 2). Oddly enough, the presence of added electron acceptor removes the difference in signal appearance induced by DCMU (Fig. 5d). At this preliminary stage it is not possible to give a full explanation to this observation. It seems reasonable, however, to assume that different paramagnetic components dominate the spectra in Figs. 5c and 5b (or 5d) i.e., more than one paramagnetic state of the oxygen-evolving center may be observed.

It is noteworthy that the main features of the multiline signal, obtained without DCMU or with DCMU and PPBQ both present, resembles those one would expect to arise from a single species consisting of an antiferromagnetically coupled pair of one Mn(III) and one Mn(IV) ion. We will here outline the derivation of the resonance condition appropriate for such a system. The presentation is more complete than that given by Dismukes and Siderer [8].

The spin hamiltonian for a pair of exchange coupled metal ions can be written [19]

$$\mathcal{H} = \mathcal{H}_1 + \mathcal{H}_2 - 2J\mathbf{S}_1 \cdot \mathbf{S}_2 + \mathbf{S}_1 \cdot D_{\mathrm{e+d}} \cdot \mathbf{S}_2 \qquad (1)$$

where the single ion spin hamiltonians

$$\mathcal{H}_i = \beta \mathbf{B} \cdot \mathbf{g}_i \cdot \mathbf{S}_i + \mathbf{S}_i \cdot \mathbf{D}_i \cdot \mathbf{S}_i + \mathbf{S}_i \cdot \mathbf{A}_i \cdot \mathbf{I}_i \qquad (i = 1, 2)$$

Here J and $D_{\mathrm{e+d}}$ represent the isotropic and anisotropic exchange interactions, respectively $D_{\mathrm{e+d}}$ can also include the dipole-dipole contribution. Introducing the total spin operator $\mathbf{S} = \mathbf{S}_1 + \mathbf{S}_2$ and assuming a strong antiferromagnetic coupling ($J < 0$, $|J| \gg g\beta B$) one arrives at an effective hamiltonian for each manifold of spin S [19]:

$$\mathcal{H}' = \beta \mathbf{B} \cdot \mathbf{g}^C \cdot \mathbf{S} + \mathbf{S} \cdot \mathbf{A}_1^C \cdot \mathbf{I}_1 + \mathbf{S} \cdot \mathbf{A}_2^C \cdot \mathbf{I}_2 + \mathbf{S} \cdot \mathbf{D}^C \cdot \mathbf{S} \qquad (2)$$

The tensors $\mathbf{g}^C$ and $\mathbf{A}_i^C$ ($i = 1,2$) are linear combinations of the single ion tensors:

$$\mathbf{g}^C = \frac{1 + C}{2} \mathbf{g}_1 + \frac{1 - C}{2} \mathbf{g}_2$$

$$\mathbf{A}_1^C = \frac{1 + C}{2} \mathbf{A}_1 , \qquad \mathbf{A}_2^C = \frac{1 - C}{2} \mathbf{A}_2$$

where the constant C is given [19] by

$$C = \frac{S_1(S_1 + 1) - S_2(S_2 + 1)}{S(S + 1)}$$

In the case of antiferromagnetic coupling between

Mn(III) ($S_1 = 2$) and Mn(IV) ($S_2 = 3/2$), we have $S = S_1 - S_2 = 1/2$ and may, therefore, (according to Kramers' theorem [20]) neglect the last term in Eqn. 2 for this doublet. Restriction to isotropic Zeeman and nuclear hyperfine interactions leads to the following reduced hamiltonian:

$$\mathcal{H}'' = g^C\beta BS_z + A_1^C(S_xI_{1x} + S_yI_{1y} + S_zI_{1z}) + A_2^C(S_xI_{2x} + S_yI_{2y} + S_zI_{2z}) \tag{3}$$

Using second-order perturbation theory together with the selection rules, $\Delta m_S = \pm 1$, $\Delta m_{Ii} = 0$ ($i = 1, 2$) [9], one finally arrives at the resonance condition

$$h\nu = g^C\beta B + A_1^C m_{I1} + A_2^C m_{I2} + \frac{(A_1^C)^2 \cdot (I_1(I_1+1) - m_{I1}^2) + (A_2^C)^2 \cdot (I_2(I_2+1) - m_{I2}^2)}{2g^C\beta B} \tag{4}$$

where $m_{Ii} = -I_i, \ldots\ldots, I_i$ ($i = 1, 2$). At constant microwave frequency, ν, this will be a quadratic equation in the line positions B. In the present case, $I_1 = I_2 = 5/2$, and Eqn. 4 will lead to 36 EPR lines, corresponding to the 6^2 different combinations of m_{I1} and m_{I2}. The positions of these lines when $g^C = 1.96$, $|A_1^C| = 17.7$ mT, $|A_2^C| = 8.2$ mT (values taken from Ref. 8) and $\nu = 9.25$ GHz are shown in Fig. 2b. The asymmetry around g^C 1.96 is due to the second-order term in Eqn. 4. There is a reasonably good agreement between the calculated spectrum and the spectra obtained in the experiments. Further theoretical work may improve the fit. The calculations imply that binuclear manganese centers in the water-splitting site may give rise to the multiline EPR signals as suggested by Dismukes and Siderer [8]. An analoguous treatment involving a tetranuclear complex, consisting of one Mn(IV) and three Mn(III) ions, and assuming isotropic Zeeman and nuclear hyperfine interactions, may result in an identical multiline spectrum. This was also pointed out by Dismukes and Siderer [8]. Thus, at this stage it is not possible to discriminate between the two structures.

Destruction of the oxygen-evolving site appears to perturb the EPR signals seen at high power levels around g 6–7 (Fig. 3). These are similar to the high-spin signals observed in some heme proteins. The marked increase in signal strength at g 4.3, typical of 'rhombic' iron, after heat treatment may also be of interest in this context, as mild heating is usually assumed to affect specifically the oxygen-evolving site [21,22]. The possible relation between the emergence of a large g 4.3 signal and the behavior of the g 6–7 signals deserves further consideration. The observations may indicate the involvement of iron in oxygen evolution, which would be consistent with the findings by Nakatani and Barber [23], who recently isolated a heme-containing protein with a possible role in water oxidation.

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