Torgerson, P. M., Drickamer, H. G., & Weber, G. (1979) *Biochemistry 18*, 3079.
van Oss, C. J., Absolom, D. R., & Bronson, P. M. (1982) *Immunol. Commun. 11*, 139.
Voss, E. W., Jr., Eschenfeld, W., & Root, R. T. (1976) *Immunochemistry 13*, 447.
Watt, R. M., & Voss, E. W., Jr. (1979) *J. Biol. Chem. 254*, 7105.

Electron Paramagnetic Resonance Properties of the S_2 State of the Oxygen-Evolving Complex of Photosystem II

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ABSTRACT: Electron paramagnetic resonance (EPR) signals arising from components in photosystem II have been studied in membranes isolated from spinach chloroplasts. A broad EPR signal at $g = 4.1$ can be photoinduced by a single laser flash at room temperature. When a series of flashes is given, the amplitude of the $g = 4.1$ signal oscillates with a period of 4, showing maxima on the first and fifth flashes. Similar oscillations occur in the amplitude of a multiline signal centered at $g \simeq 2$. Such an oscillation pattern is characteristic of the S_2 charge accumulation state in the oxygen-evolving complex. Accordingly, both EPR signals are attributed to the S_2 state. Earlier data from which the $g = 4.1$ signal was attributed to a component different from the S_2 state [Zimmermann, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160–167; Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28] are explained by the effects of cryoprotectants and solvents, which are shown to inhibit the formation of the $g = 4.1$ signal under some conditions. The $g = 4.1$ signal is less stable than the multiline signal when both signals are generated together at low temperature. This indicates that the two signals arise from different populations of centers. The differences in structure responsible for the two different EPR signals are probably minor since both kinds of centers are functional in cyclic charge accumulation and seem to be interconvertible. The difference between the two EPR signals, which arise from the same redox state of the same component (a mixed-valence manganese cluster), is proposed to be due to a spin-state change, where the $g = 4.1$ signal reflects an $S = {}^3/_2$ state and the multiline signal an $S = {}^1/_2$ state within the framework of the model of de Paula and Brudvig [de Paula, J. C., & Brudvig, G. W. (1985) *J. Am. Chem. Soc. 107*, 2643–2648]. The spin-state change induced by cryoprotectants is compared to that seen in the iron protein of nitrogenase.

The evolution of oxygen due to the photooxidation of water by higher plants, green algae, and cyanobacteria occurs by a four-step mechanism (Joliot et al., 1969). The experimental data are interpreted in a model which envisages five states, S_0, S_1, S_2, S_3, and S_4, of the oxygen-evolving complex (Kok et al., 1971). These states represent successive oxidation states, each generated by a single turnover of the photosystem II (PS II)[1] reaction center. In the model, S_0 and S_1 are stable in the dark, while S_2 and S_3 can deactivate back to S_1. S_4 reacts rapidly to S_0 in a millisecond and O_2 is evolved.

An EPR signal centered at $g = 2$ and having 19–20 hyperfine lines has been observed in broken chloroplasts following excitation with a single flash (Dismukes & Siderer, 1980). This multiline signal has been seen in thylakoids frozen under illumination (Hansson & Andréasson, 1982) or following illumination at 200 K (Brudvig et al., 1983). It has also been seen in PS II particles under the same conditions. This signal has been shown to originate from the S_2 state of the oxygen-evolving system (Dismukes & Siderer, 1980; Hansson & Andréasson, 1982; Brudvig et al., 1983; Zimmermann & Rutherford, 1984). The best evidence for this assignment is that, following a series of flashes, its amplitude oscillates with a period of 4, having maxima on the first and fifth flashes (Dismukes & Siderer, 1980; Zimmermann & Rutherford, 1984; Franzén et al., 1985). It has been suggested that this signal arises from an $S = {}^1/_2$ state of a manganese cluster (Dismukes & Siderer, 1980; Hansson & Andréasson, 1982; Brudvig et al., 1983; Dismukes et al., 1982; Andréasson et al., 1983; Hansson et al., 1984). A recent study, however, has shown that the saturation behavior and temperature dependence of this signal depend both on the length of dark adaptation before illumination and on the illumination temperature (de Paula & Brudvig, 1985; Beck et al., 1985). The multiline signal was shown to be best interpreted as originating from the interaction of an $S_1 = {}^1/_2$ state of a manganese cluster with an $S_2 = 1$ system (de Paula & Brudvig, 1985). This model also predicted the existence of an $S = {}^3/_2$ ground state.

An EPR signal at $g = 4.1$ was discovered recently, which was also attributed to a donor side component (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). It was suggested that this component was an intermediate carrier between the

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[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid (sodium salt); EPR, electron paramagnetic resonance; MES, 4-morpholineethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PS II, photosystem II; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; S_i, charge accumulation states of the O_2-evolving complex; Chl, chlorophyll.

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S states and the reaction center (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984).

In this paper, we report evidence that this $g = 4.1$ signal also arises from the S_2 state and probably originates from the $S = {}^3/_2$ ground state of the model of de Paula and Brudvig (1985).

Materials and Methods

Oxygen-evolving PS II membranes were prepared as described previously (Berthold et al., 1981; Ford & Evans, 1983), resuspended at high concentration ($\simeq$10 mg of Chl/mL) in a medium containing 400 mM sucrose, 20 mM MES, pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$, and stored at 193 K before use. For some experiments, this medium contained 50% v/v glycerol or 30% v/v ethylene glycol.

The rate of oxygen evolution in these preparations was routinely 300 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} as measured with a Clark-type O_2 electrode (Hansatech Ltd.) with 80 μM PPBQ and 1 mM ferricyanide as exogenous electron acceptors under saturating illumination.

EPR samples in calibrated quartz tubes (internal diameter 3 mm) were incubated in darkness for 30 min at 20 °C. This incubation is considered to put the oxygen-evolving complex in the resting form (Beck et al., 1985). For flash experiments, the samples were diluted to 3 mg/mL so that the flash was saturating (Zimmermann & Rutherford, 1984). In some experiments, PPBQ was added as an exogenous electron acceptor to the sample before dark adaptation to a final concentration of 0.7 mM. EDTA (1 mM) was present in each EPR sample.

EPR spectra were recorded at liquid helium temperature by using a Bruker ER 200tt X-band spectrometer fitted with an Oxford Instrument cryostat and temperature control system. The standard TE_{102} mode cavity was replaced by a Bruker ER 41 VR water-cooled cavity.

Samples were illuminated at 200 K in an unsilvered Dewar flask containing an ethanol/solid CO_2 mixture using an 800-W projector; 2 cm of water and three Calflex filters were used to cut off infrared radiation.

For flash experiments, the EPR samples were frozen in the dark, and EPR spectra were taken. The samples were then thawed and equilibrated at room temperature for about 1 min before being excited by one or more laser flashes. The flashes were provided by a Quantel Nd-YAG laser giving a 100-mJ, 15-ns pulse at 530 nm. After flash excitation, the samples were rapidly frozen (<1 s) to 200 K and then stored at 77 K. EPR spectra were recorded on the same day.

In some experiments, EPR samples were warmed to 220 K by incubating in a flow of gaseous nitrogen, the temperature of which was controlled by a Brüker B-VT-1000 temperature control system.

DCMU recrystallized from a 2-propanol solution by Dr. J. Farineau and PPBQ (Sigma Chemicals) were dissolved in Me_2SO.

Results and Discussion

Illumination of PS II membranes at 200 K results in the formation of the S_2 multiline signal (Brudvig et al., 1983) and at the same time another resonance at $g = 4.1$ (Zimmermann & Rutherford, 1984). Casey and Sauer (1984) have reported the photogeneration of an EPR signal at $g = 4.1$ upon illumination at 140 K, but they were unable to produce this signal at 200 K. To understand the origin of this discrepancy, experiments were performed by using the conditions described in the conflicting reports (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984). Figure 1a shows the EPR spectrum

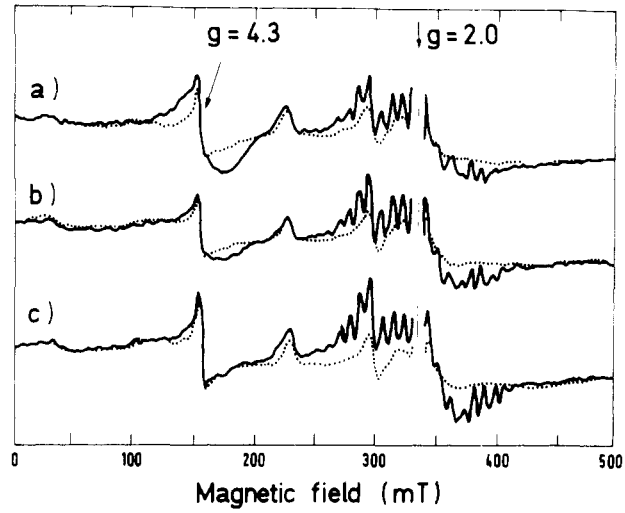

FIGURE 1: EPR spectra of dark-adapted (dotted lines) PS II particles and after 4-min illumination at 200 K (solid lines). (a) Control buffer; (b) glycerol, 50%; (c) ethylene glycol, 30%. Instrument settings: microwave frequency, 9.4 GHz; modulation amplitude, 32 G; temperature, 5 K; microwave power, 20 μW (40 dB).

of PS II particles suspended in 400 mM sucrose, 20 mM MES, pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$ and illuminated for 2 min at 200 K. As already reported under these conditions (Zimmermann & Rutherford, 1984), a broad signal at $g = 4.1$ was photoinduced in addition to the S_2 multiline signal. Figure 1b shows the spectrum of PS II particles suspended in the same medium but with 50% glycerol, as used by Casey and Sauer (1984). Under these conditions, very little $g = 4.1$ signal was photoinduced. In some experiments, no $g = 4.1$ signal was even detectable. The effect of ethylene glycol, which has also been used as a cryoprotectant in experiments done on this system [e.g., see Brudvig et al. (1983)], was also tested. Figure 1c shows the spectrum of PS II particles suspended in the presence of 30% ethylene glycol, which were illuminated for 2 min at 200 K. No $g = 4.1$ signal was photoinduced. Thus, the inability of Casey and Sauer (1984) to photoinduce the $g = 4.1$ signal upon illumination at 200 K was due to glycerol, which inhibits the formation of this signal at 200 K.

Since both glycerol and ethylene glycol inhibit the formation of the $g = 4.1$ signal under the conditions of these experiments, it seemed possible that ethanol, which is often used as a solvent for electron acceptors and inhibitors in EPR experiments, could also influence the EPR spectra. Figure 2 shows the effect of 5% ethanol on the EPR signals photoinduced at 200 K in PS II particles. The $g = 4.1$ signal is greatly diminished and nearly absent (Figure 2a). The multiline signal exhibits additional fine structure, when measured with 3.2-G modulation amplitude, and the wings of the spectrum are bigger relative to the other peaks (Figure 2b). The saturation curve of the multiline signal shows that, with 5% ethanol, this signal saturates more rapidly than in the control sample.

Incubation of PS II membranes with DCMU, which blocks electron transfer from the primary semiquinone acceptor, Q_A^-, to the secondary quinone acceptor Q_B, was reported to result in the loss of the ability to produce the $g = 4.1$ signal by illumination at 200 K although the amplitude of the S_2 multiline signal was not observed to be affected (Zimmermann & Rutherford, 1984). This result was taken as an indication that a second photochemical turnover was required to form the $g = 4.1$ signal, and this was used as an argument for ascribing the $g = 4.1$ signal to an intermediate electron carrier between the oxygen-evolving complex and the reaction center (Zimmermann & Rutherford, 1984). However, in these ex-

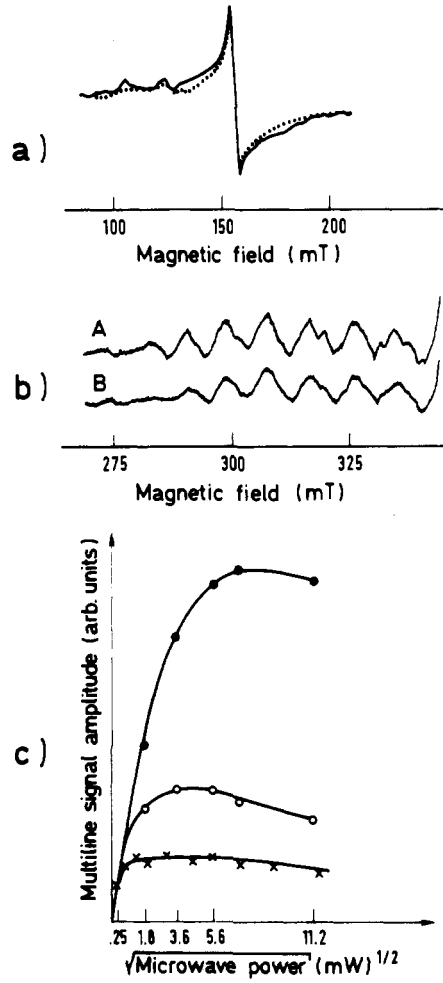

FIGURE 2: Effects of ethanol on EPR signals photoinduced in PS II membranes by 4-min illumination at 200 K. (a) Ethanol, 5%: (dotted line) dark-adapted sample; (solid line) after illumination. (b) (A) Ethanol, 5%; (B) control buffer. Spectra taken after illumination. (c) Saturation curves of the multiline signal: (open circles) control sample; (crosses) 5% ethanol; (closed circles) 50% glycerol [data taken from Zimmermann & Rutherford (1984)]. Instrument settings: (a) as in Figure 1; (b) modulation amplitude, 3.2 G; temperature, 8 K; microwave power, 2 mW; (c) modulation amplitude, 32 G; temperature, 5 K.

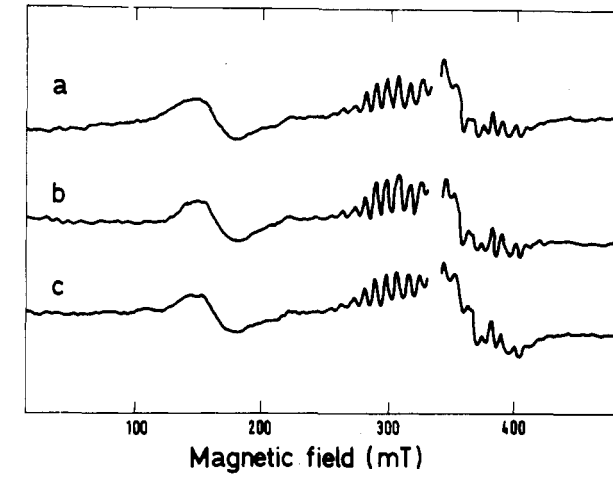

FIGURE 3: Difference spectra of PS II particles illuminated for 4 min at 200 K. The spectra are the difference between the spectra after 200 K illumination and the spectra of the same dark-adapted sample: (a) control buffer; (b) Me_2SO, 5%; (c) 1 mM DCMU in Me_2SO. Instrument settings: modulation amplitude, 20 G; temperature, 5 K; microwave power, 6.3 mW.

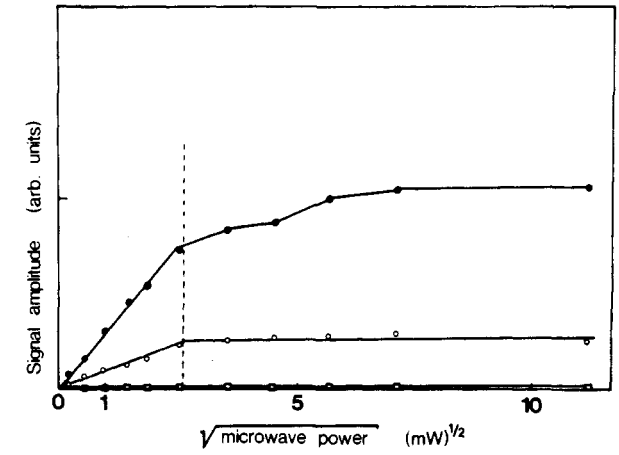

FIGURE 4: Saturation curves of the $g = 4.1$ signal photogenerated in PS II particles by 4-min illumination at 200 K. (Closed circles) control buffer; (open circles) 5% ethanol; (open squares) 50% glycerol. Instrument settings: modulation amplitude, 20 G; temperature, 5 K.

periments, DCMU was dissolved in ethanol. It is shown here that the presence of ethanol itself leads to the loss of photoinduction of the $g = 4.1$ signal at 200 K. Thus, the DCMU experiment was redone with another solvent. Figure 3b shows the effect of 5% Me_2SO on the $g = 4.1$ and multiline signals generated by a 2-min illumination at 200 K. Comparison with Figure 3a shows that the size and shape of both signals are almost identical with or without Me_2SO. Figure 3c shows that DCMU dissolved in Me_2SO at a concentration of 1 mM, which is more than sufficient to block the reoxidation of Q_A^- in all the centers, has little effect on the size and shape of either the $g = 4.1$ or the multiline signals. Thus, the DCMU effect reported earlier (Zimmermann & Rutherford, 1984) was in fact mainly due to ethanol, and the inhibitory properties of DCMU are not responsible for the loss of the $g = 4.1$ signal.

The diminution or loss of the ability to observe the $g = 4.1$ signal under some circumstances could be due either to changes in the saturation properties of this signal under these circumstances or to a diminution or loss of the spin concentration of the species responsible for the $g = 4.1$ signal. Figure 4 shows the saturation curve of the residual $g = 4.1$ signal produced in the presence of ethanol, compared to that in the control sample. In both cases, the $g = 4.1$ signal saturates at about 8.5 mW at 5 K. Figure 4 shows also that the absence of the $g = 4.1$ signal in a sample containing 50% glycerol is not due to a saturation effect. Thus, the loss of the $g = 4.1$ signal in the experiments reported above is due to the loss of the species giving rise to the $g = 4.1$ signal.

It was shown previously that excitation of PS II particles with one laser flash at room temperature resulted in the formation of the S_2 multiline signal and that no $g = 4.1$ signal was formed (Zimmermann & Rutherford, 1984). This was taken as evidence that the $g \approx 4.1$ signal arose from a component different from S_2 (Zimmermann & Rutherford, 1984). This experiment was done in the presence of PPBQ, an exogenous electron acceptor, which keeps the plastoquinone pool oxidized. However, PPBQ was dissolved in ethanol. This experiment was repeated in the absence of an electron acceptor. Figure 5 shows that excitation of PS II particles with one laser flash at room temperature results in the photogeneration of both the $g = 4.1$ and the multiline signals with amplitudes similar to those seen in samples illuminated at 200 K. A period of illumination at 200 K given to a sample which had received a flash at 20 °C produced no further increase in either the $g = 4.1$ or the multiline signals (Figure 5). In the presence of 5% ethanol, no $g = 4.1$ signal is photoinduced by one flash.

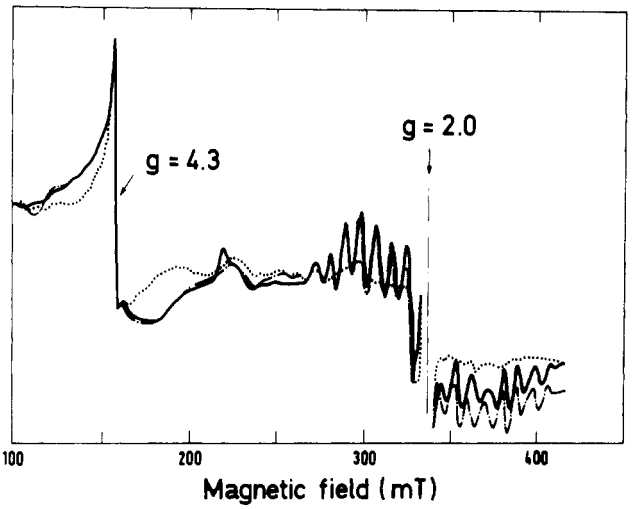

FIGURE 5: EPR spectra of dark-adapted PS II particles (dotted line) excited with one laser flash at room temperature (dashed line) and with a further 4-min illumination at 200 K (solid line). Instrument settings: modulation amplitude, 32 G; temperature, 8 K; microwave power, 2 mW.

The fact that one flash at room temperature results in the photogeneration of the $g = 4.1$ signal indicates that the species responsible for the $g = 4.1$ signal is S_2 itself or an electron donor on a side path which competes with S_1 donation to the reaction center or perhaps functions instead of S_1 in damaged centers.

Figure 6a shows the EPR spectra of PS II particles excited with a series of flashes at room temperature in the presence of PPBQ (0.8 mM) dissolved in Me_2SO. The amplitude of the $g = 4.1$ signal is observed to oscillate with a period of 4, having maxima on the first and fifth flashes. The oscillation behavior closely matches that of the multiline signal (Figure 6). This result indicates that the $g = 4.1$ resonance originates, together with the multiline signal, from the S_2 state of the oxygen-evolving system.

An observation that led to the conclusion that the S_2 multiline signal and the $g = 4.1$ signal arose from different components was that the $g = 4.1$ species generated in PS II particles by illumination at 200 K decayed more quickly than the multiline species when the sample was warmed to 200 K (Zimmermann & Rutherford, 1984). This experiment was redone in the presence of DCMU (in the absence of ethanol), so that the Q_A^- to Q_B electron transfer could not occur upon illumination or upon warming. The results of such an experiment are depicted in Figure 7.

Illumination of PS II membranes for 2 min at 200 K in the presence of 100 μM DCMU results in the photogeneration of the multiline signal, the $g = 4.1$ signal, and the Q_A^-Fe signal (Nugent et al., 1981; Rutherford & Mathis, 1983). Warming the sample to 220 K for various times results in the decrease of the three signals: after 8 min at 220 K, the remaining $g = 4.1$ signal is 57%, the remaining multiline signal is 92%, and the Q_A^-Fe signal is 83% of the maximum size photoinduced at 200 K, respectively. Interference with the measurements of the Q_A^-Fe signal by the multiline signal was minimized by increasing the power and lowering the temperature. However, overlap of the two signals makes the above values only approximate. Illumination of the sample for 2 min at 200 K after this treatment regenerates the three signals close to their original amplitudes. It is clear that the $g = 4.1$ signal decreases more rapidly in this experiment than do the multiline or the Q_A^-Fe signals. The faster decrease of the $g = 4.1$ signal upon warming could correspond to the conversion of the $g = 4.1$ signal to the multiline signal. Such an interconversion was reported by Casey and Sauer (1984) when glycerol-containing samples which had been illuminated at 140 K were warmed in the dark. This effect may occur to a small extent and could be responsible for the slight increase in the multiline signal which is sometimes observed after warming for short times (Zimmermann & Rutherford, 1984). However, since the Q_A^-Fe signal decreases upon warming and since a second

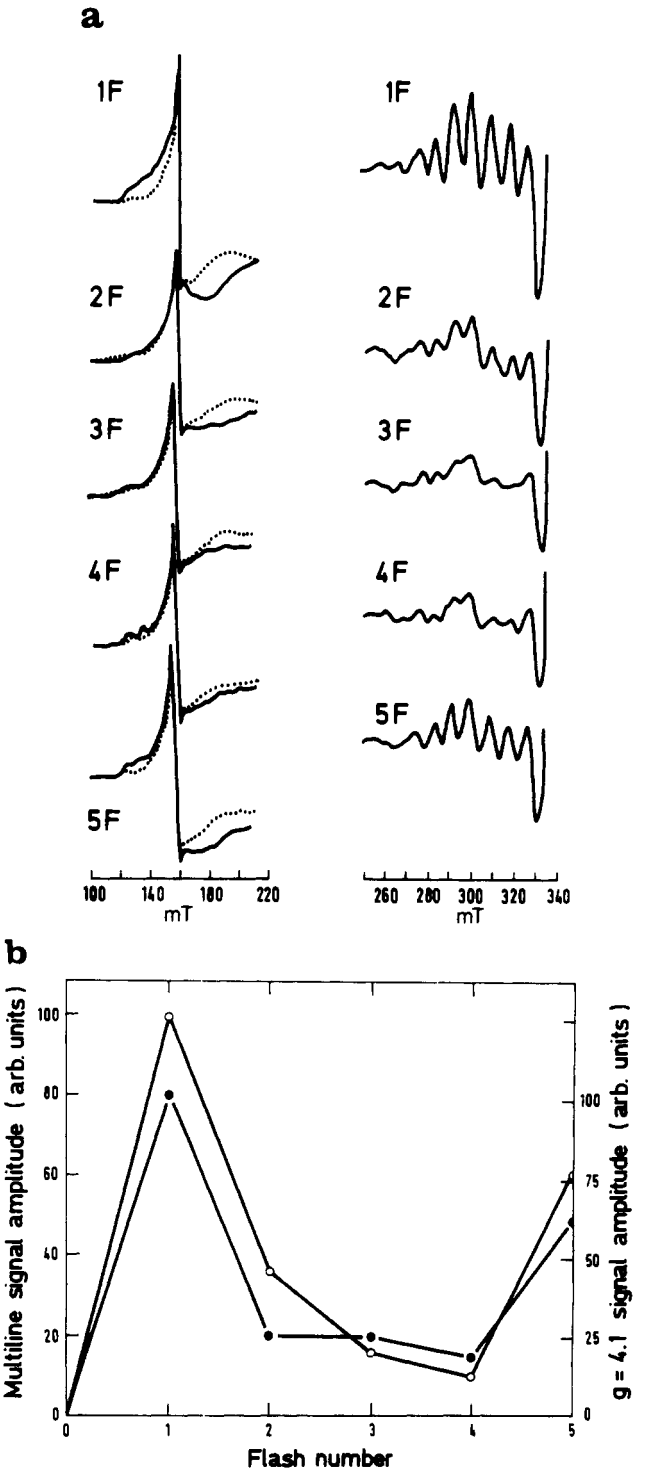

FIGURE 6: (a) EPR spectra of PS II membranes excited with a series of laser flashes at room temperature. Instrument settings: temperature, 8 K; microwave power, 2 mW; modulation amplitude, 32 G. Multiline signals are expanded approximately 1.4-fold relative to the $g = 4.1$ signals. (b) Amplitude of the multiline signal (open circles) and of the $g = 4.1$ signal (closed circles) detected after a series of flashes at room temperature. Instrument settings as in Figure 6a except that the microwave power was 6.3 mW.

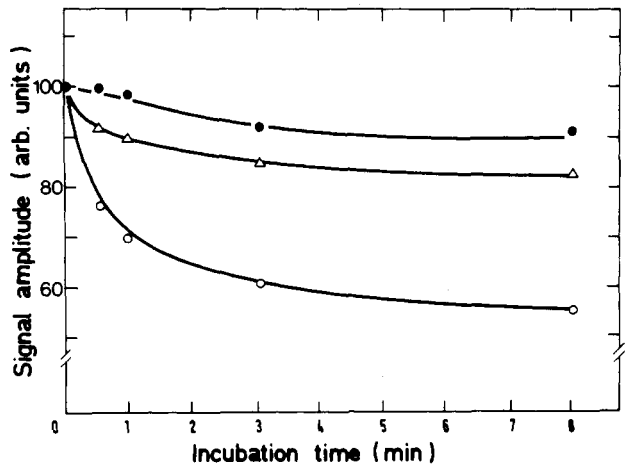

FIGURE 7: Amplitudes of EPR signals generated in PS II particles by 4-min illumination at 200 K and by further incubation for various times at 220 K. Closed and open circles, multiline and $g = 4.1$ signals, respectively, using the following instrument settings: temperature, 8 K; modulation amplitude, 32 G; microwave power, 2 mW (average of two spectra). Triangles represent the Q_A^-Fe signal at $g = 1.82$ measured at a temperature of 3.8 K and a microwave power of 50 mW.

illumination at 200 K regenerates both the $g = 4.1$ signal and the Q_A^-Fe signal to their original amplitudes, the decay of the $g = 4.1$ signal is attributed to a recombination back-reaction.

These results indicate that the two kinds of EPR signals, the multiline and the $g = 4.1$ signals, arise from different populations of centers. The centers in the S_2 state which give rise to the $g = 4.1$ signal appear to be more unstable than those which give rise to the multiline signal. Judging from the extent of decay of the three signals upon warming, the $g = 4.1$ centers represent approximately 25% of the centers.

The effect of ethylene glycol as an inhibitor of formation of the $g = 4.1$ signal is shown to be reversible in Figure 8 (the reversibility of the effect of ethanol and glycerol on the $g = 4.1$ signal has not yet been studied). PS II membranes suspended with 30% ethylene glycol, a treatment which inhibits the formation of the $g = 4.1$ signal (Figure 1c), were washed 3 times in 50 mM MES pH 6.0, 15 mM NaCl, and 10 mM $MgCl_2$ and centrifuged at 35000g for 20 min. The resulting pellet was suspended in 400 mM sucrose, 20 mM MES, pH 6.0, 5 mM $MgCl_2$, and 15 mM NaCl. A control sample was prepared by the same treatment, except that buffers contained no ethylene glycol. Figure 8 shows that illumination at 200 K of an ethylene glycol treated and washed sample results in the photogeneration of the $g = 4.1$ signal to approximately the same extent as that in the control sample. Furthermore, it is of note that in the presence of ethylene glycol, the amplitude of the multiline signal is slightly but consistently bigger than that without ethylene glycol, when measured at low microwave powers, i.e., when the signal is not saturated (Figure 1c), thus showing an increase in spin concentration. Thus, it is concluded that those centers, which give rise to the $g = 4.1$ signal when in the S_2 state, seem to be converted to centers giving rise to the multiline signal upon addition of ethylene glycol and that this effect is reversible.

CONCLUSIONS

Glycerol and ethylene glycol inhibit the photogeneration of the $g = 4.1$ signal at 200 K, and ethanol results in a loss of approximately 70% of this signal. In addition, it was reported earlier that the presence of glycerol changes the saturation properties of the multiline signal: glycerol was shown to shorten the relaxation time of the multiline species (Zimmermann & Rutherford, 1984). However, the presence of the $g = 4.1$ signal and the saturation properties of the multiline signal are not necessarily directly related, since the presence of ethanol, which also results in a significant decrease in the photogenerated $g = 4.1$ signal, has the opposite effect on the saturation of the multiline signal (Figure 2c). Changes in the shape and saturation behavior of the multiline signal have also been observed in the presence of ethylene glycol. However, these changes are less marked than in the presence of glycerol or ethanol, and the different lines of the spectrum seem to saturate differently. An extensive study on the saturation of the multiline signal in the presence of ethylene glycol has been reported recently (de Paula & Brudvig, 1985), and it was shown that the saturation behavior of the multiline signal was dependent upon the length of dark adaptation prior to illumination and upon the temperature of this illumination.

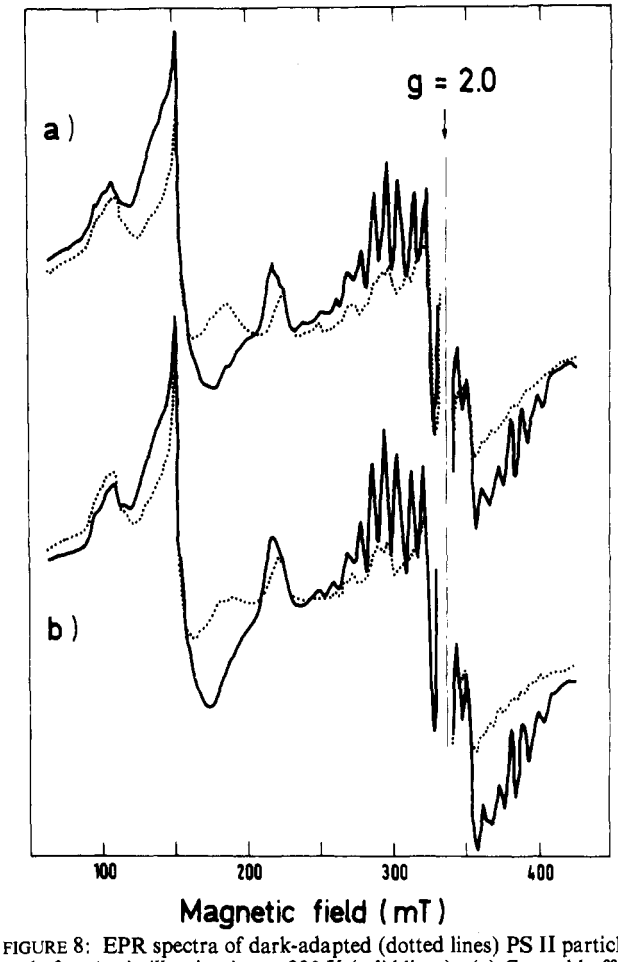

FIGURE 8: EPR spectra of dark-adapted (dotted lines) PS II particles and after 4-min illumination at 200 K (solid lines). (a) Control buffer; (b) membranes suspended with 30% ethylene glycol and then washed and resuspended with control buffer (see text for details). Instrument settings: modulation amplitude, 32 G; temperature, 8 K; microwave power, 2 mW (20 dB).

Small amounts of alcohols have been reported to affect the line shape of the EPR signal of high-spin Fe^{3+} in soybean lipoxygenase 1. The presence of alcohols was reported to cause a shift to an axial type of spectrum (Slappendel et al., 1982a). It was concluded that alcohol modified the structure of the environment of the iron atom (Slappendel et al., 1982a) through direct bonding of alcohol in the vicinity of iron (Slappendel et al., 1982b). Similarly, the effect of alcohol on the photoinduced $g = 4.1$ signal may be explained by changes in the environment of the metal center.

On the basis of a temperature dependence and power saturation study of the multiline signal, de Paula and Brudvig

(1985) recently proposed a model in which the multiline signal arose from an $S = {}^1/_2$ state of a manganese dimer weakly coupled to an $S = 1$ system. In the model, the multiline signal arose from an $S = {}^1/_2$ excited state, and the existence of an $S = {}^3/_2$ ground state was predicted.

Intermediate spin states ($S = {}^3/_2$) (Maltempo & Moss, 1976) have been observed in heme proteins [e.g., see Maltempo et al. (1974)]. Such states give rise to a characteristic EPR signal with $g_\perp \simeq 4.0$ and $g_\parallel \simeq 2.0$. If the photoinduced $g = 4.1$ signal arises from such a magnetic state, the $g_\parallel$ resonance would occur in the $g \simeq 2$ region and would thus be difficult to detect because of the presence of other signals. In addition, the orientation data on the $g = 4.1$ signal reported by Rutherford (1985) could be accounted for by some rhombic distortion of the crystal field. It is thus reasonable to assign the $g = 4.1$ signal to the $S = {}^3/_2$ ground state of the model of de Paula and Brudvig (1985). Indeed, it is demonstrated in the present paper that both the $g = 4.1$ and multiline signals arise from the same component in different photochemical centers. The origin of the $S = 1$ system in the model of de Paula and Brudvig (1985) is not known, but an obvious candidate is another manganese dimer since it is thought from other lines of evidence that four manganese atoms make up the oxygen-evolving complex (Amesz, 1983).

The effects of cryoprotectants on the two EPR signals reported here are of particular interest when compared to the properties of the Fe protein of nitrogenase. The native form of this enzyme has been recently reported to be heterogeneous, giving rise to two EPR signals, one attributed to an $S = {}^1/_2$ state and the other to an $S = {}^3/_2$ state (Hagen et al., 1985; Lindahl et al., 1985). The $S = {}^3/_2$ signal is converted into the $S = {}^1/_2$ state by addition of ethylene glycol. The two different forms do not reflect differences in activity and are thought to reflect very slight structural differences. This phenomenon is strikingly similar to those reported here for the oxygen-evolving complex and may reflect analogous spin-state changes.

In addition, Lindahl et al. (1985) have related the spin-state variability (i.e., mixture of $S = {}^1/_2$ and $S = {}^3/_2$ states) of the nitrogenase Fe protein to the fact that the iron–sulfur cluster links different protein subunits. It is of note that in the oxygen-evolving complex, the extrinsic 33-kilodalton polypeptide has been reported to be isolated either with (Abramowicz & Dismukes, 1984; Yamamoto et al., 1984) or without (Franzén & Andréasson, 1984; Miyao & Murata, 1984; Ono & Inoue, 1984) manganese bound to it. This has led to models in which the functional manganese is associated with two different proteins (Zimmermann & Rutherford, 1985; Dismukes, 1985). Thus, we speculate that the sensitivity of the EPR signals to the local environment reported here may be due to the manganese cluster bridging between two polypeptides.

De Paula et al. (1986) recently performed a systematic study of low-temperature photochemistry in which all the photogenerated species were quantified under conditions similar to those used by Casey and Sauer (1984), i.e., in the presence of a cryoprotectant. Results similar to those of Casey and Sauer (1984) on the formation of the $g = 4.1$ signal were obtained. However, it was proposed that both signals might arise from the S_2 state of the O_2-evolving complex. The data presented here substantiate this idea.

In this paper, evidence is provided that in a small proportion of centers (those giving rise to the $g = 4.1$ signal) S_2 deactivates by recombination with Q_A^- at 220 K. This recombination reaction is probably the origin of the so-called Zv band of thermoluminescence. Many of the properties of the Zv band, in particular its sensitivity to cryoprotectants and ethanol and its flash dependence (Vass et al., 1984), are strikingly similar to those predicted from the EPR work reported here.

Here, we attribute the $g = 4.1$ signal to the S_2 state of a subpopulation of the centers in which the manganese cluster exists in a slightly different structural environment. It has been reported (Casey & Sauer, 1984; de Paula et al., 1985) that in the presence of cryoprotectants, illumination at 140 K results in the formation of the $g = 4.1$ signal and that warming to 200 K converts the $g = 4.1$ signal to the multiline signal. Similar results have been observed in the absence of cryoprotectants (unpublished results). These observations can be interpreted as a temperature-dependent conformational difference around the manganese cluster. The data reported here indicate that in the absence of cryoprotectants a small proportion of the centers maintains the environment of the manganese cluster responsible for the $g = 4.1$ signal even at room temperature.

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Registry No. $HOCH_2CH(OH)CH_2OH$, 56-81-5; $HO(CH_2)_2OH$, 107-21-1; EtOH, 64-17-5.

REFERENCES

Abramowicz, D. A., & Dismukes, G. C. (1984) *Biochim. Biophys. Acta 765*, 318–328.

Amesz, J. (1983) *Biochim. Biophys. Acta 726*, 1–12.

Andréasson, L. E., Hansson, Ö., & Vänngård, T. (1983) *Chem. Scr. 21*, 71–74.

Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1985) *Biochemistry 24*, 3035–3043.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.

Brudvig, G. W., Casey, J. L., & Sauer, K. (1983) *Biochim. Biophys. Acta 723*, 366–371.

Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28.

de Paula, J. C., & Brudvig, G. W. (1985) *J. Am. Chem. Soc. 107*, 2643–2648.

de Paula, J. C., Innes, J. B., & Brudvig, G. W. (1985) *Biochemistry 24*, 8114–8120.

Dismukes, G. C. (1985) *Photochem. Photobiol. 43*, 99–115.

Dismukes, G. C., & Siderer, Y. (1980) *FEBS Lett. 121*, 78–80.

Dismukes, G. C., Ferris, K., & Watnick, P. (1982) *Photobiochem. Photobiophys. 3*, 243–256.

Ford, R. C., & Evans, M. C. W. (1983) *FEBS Lett. 160*, 159–164.

Franzén, L. G., & Andréasson, L. E. (1984) *Biochim. Biophys. Acta 765*, 166–170.

Franzén, L. G., Hansson, Ö., & Andréasson, L. E. (1985) *Biochim. Biophys. Acta 808*, 171–179.

Hagen, W. R., Eady, R. R., Dunham, W. R., & Haaker, H. (1985) *FEBS Lett. 189*, 250–254.

Hansson, Ö., & Andréasson, L. E. (1982) *Biochim. Biophys. Acta 679*, 261–268.

Hansson, Ö., Andréasson, L. E., & Vänngård, T. (1984) *Advances in Photosynthesis Research*, Vol. I, pp 307–310, M. Nijhoff, The Hague, The Netherlands.

Joliot, P., Barbieri, G., & Chabaud, R. (1969) *Photochem. Photobiol. 10*, 309–329.

Kok, B., Forbush, B., & McGloin, P. (1971) *Photochem. Photobiol. 11*, 457–475.

Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. H., & Münck, E. (1985) *J. Biol. Chem. 260*, 11160–11173.
Maltempo, M. M., & Moss, T. H. (1976) *Q. Rev. Biophys. 9*, 181–215.
Maltempo, M. M., Moss, T. H., & Cusanovich, M. A. (1974) *Biochim. Biophys. Acta 342*, 290–305.
Miyao, M., & Murata, N. (1984) *FEBS Lett. 170*, 350–354.
Nugent, J. H. A., Diner, B. A., & Evans, M. C. W. (1981) *FEBS Lett. 124*, 241–244.
Ono, T. A., & Inoue, Y. (1984) *FEBS Lett. 164*, 255–260.
Rutherford, A. W. (1985) *Biochim. Biophys. Acta 807*, 189–201.
Rutherford, A. W., & Mathis, P. (1983) *FEBS Lett. 154*, 328–334.
Slappendel, S., Aasa, R., Malmström, B. G., Verhagen, J., Veldink, G. A., & Vliegenthart, J. F. G. (1982a) *Biochim. Biophys. Acta 708*, 259–265.
Slappendel, S., Aasa, R., Falk, K. E., Malmström, B. G., Vänngård, T., Veldink, G. A., & Vliegenthart, J. F. G. (1982b) *Biochim. Biophys. Acta 708*, 266–271.
Vass, E., Rozsa, Zs., & Demeter, S. (1984) *Photochem. Photobiol. 40*, 407–411.
Yamamoto, Y., Shinkai, H., Isogai, Y., Matsumura, K., & Nishimura, M. (1984) *FEBS Lett. 175*, 429–432.
Zimmermann, J.-L., & Rutherford, A. W. (1984) *Biochim. Biophys. Acta 767*, 160–167.
Zimmermann, J.-L., & Rutherford, A. W. (1985) *Physiol. Veg. 23*, 425–434.

Effects of Vanadate on the Rotational Dynamics of Spin-Labeled Calcium Adenosinetriphosphatase in Sarcoplasmic Reticulum Membranes†

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ABSTRACT: We have studied the effects of vanadate on the rotational motion of the calcium adenosinetriphosphatase (Ca-ATPase) from sarcoplasmic reticulum (SR), using saturation-transfer electron paramagnetic resonance (ST-EPR). Vanadate has been proposed to act as a phosphate analogue and produce a stable intermediate state similar to the phosphoenzyme. This study provides evidence about the physical state of this intermediate. In particular, since ST-EPR provides a sensitive measure of microsecond protein rotational mobility, and hence of protein–protein association, these studies allowed us to ask (a) whether the vanadate-induced protein association observed in electron micrographs of SR vesicles also occurs under physiological (as opposed to fixed, stained, or frozen) conditions and (b) whether vanadate-induced changes in protein association also occur under conditions sufficient for enzyme inhibition but not for the production of large arrays detectable by electron microscopy (EM). At 5 mM decavanadate, a concentration sufficient to crystallize the ATPase on greater than 90% of the membrane surface area in EM, ST-EPR showed substantial immobilization of the spin-labeled protein, indicating protein–protein association in the unstained vesicles. Conventional EPR spectra of lipid probes showed that lipid hydrocarbon chain mobility is unaffected by decavanadate-induced protein crystallization in SR, suggesting that changes in protein–protein contacts do not involve the lipid hydrocarbon region. At 5 mM monovanadate, a concentration sufficient to inhibit the ATPase but not to form crystals detectable by EM, no changes were observed in ST-EPR or conventional EPR spectra of either protein or lipid. In summary, these results indicate that decavanadate induces extensive self-association of the Ca-ATPase but that monovanadate inhibits without a change in protein mobility, thus indicating no change in oligomeric state. If monovanadate produces an analogue of a phosphoenzyme state that is important in the Ca-ATPase cycle, this phosphoenzyme has the same oligomeric state as the unphosphorylated enzyme. Alternatively, if decavanadate produces a phosphoenzyme analogue, the present results suggest that protein association is increased in the phosphoenzyme state.

A central question in studies on the calcium adenosinetriphosphatase (Ca-ATPase) of sarcoplasmic reticulum (SR)[1] concerns the possible oligomeric state of the enzyme. This question is important not only for determination of the enzyme's native state but also for determination of the role protein–protein interactions may play in active calcium transport. Therefore, in order to gain an understanding of the transport mechanism, it is necessary to know whether the enzyme exists as a monomer or oligomer in its resting state, whether this oligomeric state is important for the cycle, and whether the oligomeric state changes during the cycle.

The Ca-ATPase migrates as a 115-kDa monomer on polyacrylamide gels in the presence of sodium dodecyl sulfate,

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[1] Abbreviations: SR, sarcoplasmic reticulum; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; EM, electron microscopy; V_i, vanadate; MSL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; 5-SASL and 16-SASL, stearic acid lipid probes with the doxyl group at the 5- and 16-positions, respectively; SRB, sarcoplasmic reticulum buffer; CI, crystallization index; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography.

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