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EPR STUDIES OF THE OXYGEN-EVOLVING ENZYME OF PHOTOSYSTEM II

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The light-induced EPR multiline signal is studied in O_2-evolving PS II membranes. The following results are reported: (1) Its amplitude is shown to oscillate with a period of 4, with respect to the number of flashes given at room temperature (maxima on the first and fifth flashes). (2) Glycerol enhances the signal intensity. This effect is shown to come from changes in relaxation properties rather than an increase in spin concentration. (3) Deactivation experiments clearly indicate an association with the S_2 state of the water-oxidizing enzyme. A signal at $g = 4.1$ with a linewidth of 360 G is also reported and it is suggested that this arises from an intermediate donor between the S states and the reaction centre. This suggestion is based on the following observations: (1) The $g = 4.1$ signal is formed by illumination at 200 K and not by flash excitation at room temperature, suggesting that it arises from an intermediate unstable under physiological conditions. (2) The formation of the $g = 4.1$ signal at 200 K does not occur in the presence of DCMU, indicating that more than one turnover is required for its maximum formation. (3) The $g = 4.1$ signal decreases in the dark at 220 K probably by recombination with Q_A^-Fe. This recombination occurs before the multiline signal decreases, indicating that the $g = 4.1$ species is less stable than S_2. (4) At short times, the decay of the $g = 4.1$ signal corresponds with a slight increase in the multiline S_2 signal, suggesting that the loss of the $g = 4.1$ signal results in the disappearance of a magnetic interaction which diminishes the multiline signal intensity. (5) Tris-washed PS II membranes illuminated at 200 K do not exhibit the signal.

Introduction

Illumination of intact thylakoids with a single flash at room temperature [1,2], with continuous light during freezing [3], or at 200 K [4], gives rise to an EPR multiline signal, thought to arise from the S_2 Kok state [5,6]. A number of groups has seen the signal in PS II particles from spinach chloroplasts (Refs. 7 and 8; Damoder, R. and Dismukes, G.C., personal communication) or from a cyanobacterium [9] under the same conditions. From the comparison of the signal with those of synthetically prepared ions [10,11], it has been suggested that the observed spectrum arises from a cluster of two or perhaps four manganese ions [12,13], in agreement with the present knowledge of the role of manganese in oxygen evolution of photosynthesis (reviewed in Ref. 14). In this study, we used an O_2-evolving PS II preparation to provide further evidence that the EPR multiline signal arises from the S_2 state, by confirming that the signal indeed oscillates with a period of 4 having maxima after one and five flashes, and by showing that the decay of the flash-induced signal matches the deactivation of S_2 as previously reported in

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; Q_AFe, primary semiquinone-iron complex, Chl, chlorophyll.

this kind of preparation [15].

We have also investigated the effect of glycerol in enhancing the signal intensity and show that this is due to a glycerol-induced change in the saturation characteristics of the S_2 signal.

We report the existence of a $g = 4.1$ signal and some experiments which indicate that it is an intermediate carrier between the S states and the reaction centre.

A brief report of an EPR signal in this region has recently been published by Casey and Sauer [16], who from different evidence attributed this signal to a precursor to S_2.

Materials and Methods

O_2-evolving PS II particles were prepared from spinach as previously described [17] but with the modifications described in Refs. 18 and 19. The membranes were resuspended at high concentration (approx. 12 mg Chl/ml) in a medium comprising 400 mM sucrose/20 mM Mes (pH 6.0)/15 mM NaCl/5 mM $MgCl_2$ (medium 1) and used the same day or stored at 193 K before use. No difference in the photochemistry was observed between the fresh and frozen preparations.

Tris-washed PS II particles were prepared by resuspension in room light in 0.8 M Tris-NaOH (pH 8.2) and incubation in darkness for 40 min before pelleting. The pellets were resuspended in a medium comprising 50 mM Mes (pH 6.0)/15 mM NaCl/10 mM $MgCl_2$. The resuspension was centrifuged for 30 min at $35\,000 \times g$. The final pellets were resuspended at high concentration in medium 1, as for normal membranes.

For flash experiments, the samples were diluted until the flash was saturating (approx. 4 mg Chl/ml). This was verified by monitoring the size of the multiline signal induced by the laser flash attenuated by neutral density filters. For some experiments where flash excitation was not used, samples with a higher concentration of chlorophyll were used (see legends to figures).

EPR samples in calibrated quartz tubes were incubated in darkness for 10 min at 20 °C in the presence of EDTA (2 mM) and phenyl-*p*-benzoquinone (0.7 mM) as an electron acceptor before being frozen to 77 K. In some experiments, glycerol was mixed to the PS II preparation just before making the EPR samples, so that the final concentration was 50%.

EPR spectra were recorded at liquid helium temperature using a Bruker ER-200t-*X*-band EPR spectrometer and an Oxford Instruments cryostat and temperature control system. A Tracor-Northern 1710 apparatus was used, where necessary, for averaging and substraction of spectra (see legends).

Continuous illumination at 200 K was provided by an 800 W projector in a non-silvered dewar flask containing an ethanol/solid CO_2 mixture.

A Quantel Nd-YAG Laser providing a 80 mJ and 15 ns laser flash at 530 nm was used in the flash experiments. Flashes were given either at room temperature or at 0 °C in a bath containing ice/water. Samples were rapidly frozen (less than 2 s) to 200 K after flash excitation and then stored at 77 K before EPR spectra were obtained.

In some experiments, the frozen samples were warmed to temperatures between 200 and 273 K by incubating for various times in darkness in a flow of gaseous N_2, the temperature was controlled by a Bruker B-VT-1000 temperature control system.

Phenyl-*p*-benzoquinone was purchased from Sigma Chemicals and dissolved in ethanol (40 mM). DCMU (Sigma Chemicals) was recrystallized in isopropanol and stored as a 20 mM stock.

Results and Discussion

The multiline signal

Fig. 1a shows a difference spectrum (light minus dark) of a sample of PS II membranes illuminated for 6 min at 200 K. As expected in these conditions [4], a large, well-resolved multiline signal is induced. The improved resolution obtained here by using purified PS II particles allows at least 19 lines to be resolved in this spectrum. This signal has been attributed to the second oxidizing equivalent of the water-splitting enzyme (the 'S_2 state'). At this temperature (15 K), the $g < 2$ side of the spectrum is modified by two underlying signals: firstly, a signal with features at $g = 1.82$ and $g = 1.65$ arising from the primary quinone acceptor of PS II, Q_A^-Fe [20,21,18]; and secondly, a signal at $g = 1.9$, which has been attributed to an alternative resonance form of Q_A^-Fe [7,22]. Even taking

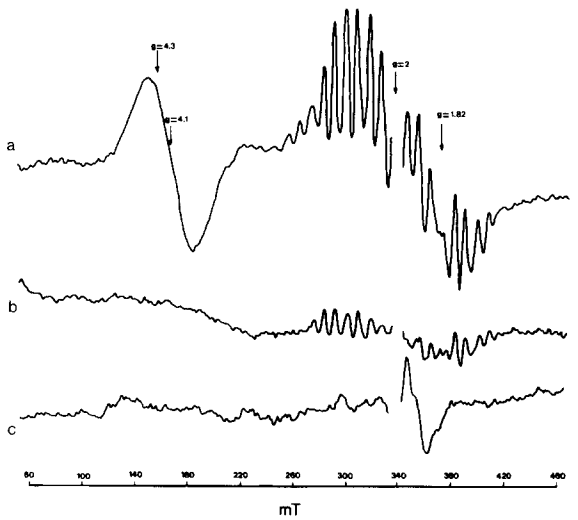

Fig. 1. Difference spectra (light minus dark) of PS II membranes. (a) [Chl] ≈ 12 mg/ml, after illumination for 6 min at 200 K; (b) [Chl] ≈ 4 mg/ml, after excitation by one flash at 20 °C; (c) [Chl] ≈ 8 mg/ml, Tris-washed membranes illuminated for 6 min at 200 K. Instrument settings: temperature, 15 K; microwave power, 31.5 mW; microwave frequency, 9.46 GHz; modulation amplitude, 20 G. Average of two spectra.

the two Q_A^-Fe signals into account, it can be seen that the multiline signal appears on top of a 750 G wide signal centered at $g \approx 2$. It is not known whether this signal arises from S_2, with the 19 lines as a hyperfine structure, or comes from another component. This suggestion has also been made by other authors (Brudvig, G.W. and Hansson, Ö, personal communication).

Of note in Fig. 1a is also the presence of a big, structureless 360 G wide signal at $g = 4.1$. A signal in this region has been observed by a number of groups (Zimmermann, J.L. and Rutherford, A.W., unpublished data; Brudvig, G.W. and Hansson, Ö, personal communication) and a brief report of such a signal has already been published [16]. Some data pertinent to the origin of the $g = 4.1$ signal are presented below.

Oscillations

In the first report of the multiline signal in thylakoids, it was shown that the signal had a flash dependence that indicated its origin from S_2 [1]. No confirmation of this oscillatory behaviour has since been published.

Indeed other groups were unable to observe such an oscillation pattern. However, by using O_2-evolving PS II particles at an appropriate concentration, in the presence of an electron acceptor (phenyl-*p*-benzoquinone) and by using a powerful laser flash, we have been able to routinely obtain well-resolved oscillation patterns with maxima at the first and fifth flashes. Fig. 2 shows the results of a typical experiment. This is good confirmation of the original observation of Dismukes and Siderer [1] and strongly enforces the idea that the signal arises from S_2.

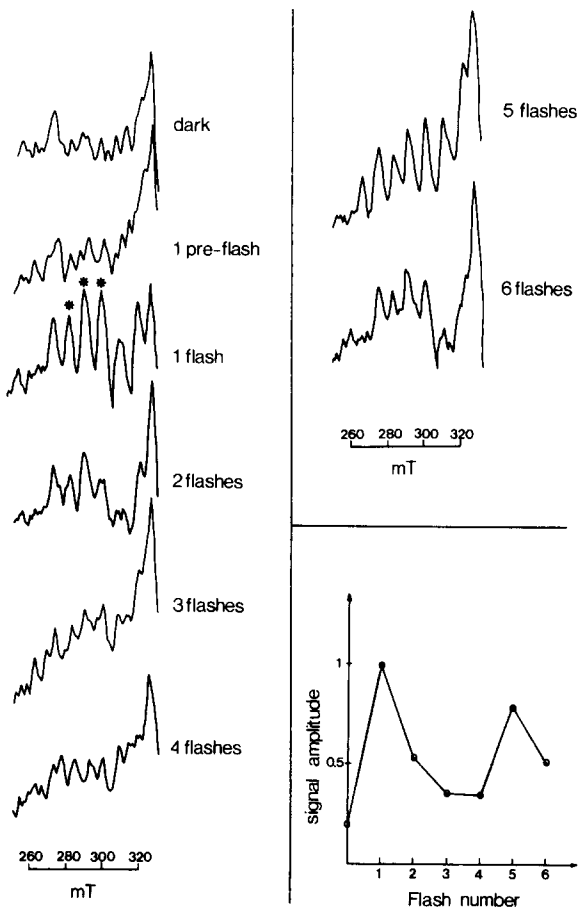

Fig. 2. Multiline signals photoinduced by a series of flashes. Samples of PS II membranes ([Chl] ≈ 3.9 mg/ml) were given one preflash at 20 °C, left to incubate in darkness for 15 min and illuminated by *n* flashes at 0 °C. Instrument settings: temperature, 8 K; microwave power, 31.5 mW; frequency, 9.44 GHz; modulation amplitude, 25 G. The amplitude of the signal was measured as the height of the three downfield peaks marked *.

Effect of glycerol

Brudvig et al. [4] found that samples containing glycerol exhibit a larger multiline signal when illuminated at 200 K and that a flash at room temperature induces the signal only when glycerol is present. Other groups did their experiments with glycerol [1,2,4,9] and until now, the involvement of glycerol in the multiline species has remained obscure.

Fig. 3a shows the effect of glycerol on the amplitude of the multiline signal induced by illumination at low temperature or by a single flash at room temperature. Unlike Brudvig et al. [4], we were able to observe the signal in a sample containing no glycerol that had been flashed at room temperature. Moreover, in samples containing glycerol, a signal of the same amplitude is induced by flash excitation or continuous illumination. In the sample not containing glycerol, the size of the signal is approximately half of that observed with glycerol with continuous illumination and slightly less with a saturating flash.

A flash pattern similar to that in Fig. 2 is obtained with glycerol (not shown).

The effect of glycerol on the amplitude of the multiline signal has been further studied by measuring its power saturation. Fig. 3b shows the dependence of its amplitude with the microwave power in the presence and in the absence of glycerol. Although the S_2 signal in the presence of glycerol is larger than that in the absence of glycerol at high power (6 dB, 5 mW), at low power, the signals have almost the same size (35 dB, 62,5 μW) (Fig. 3c). These results indicate that

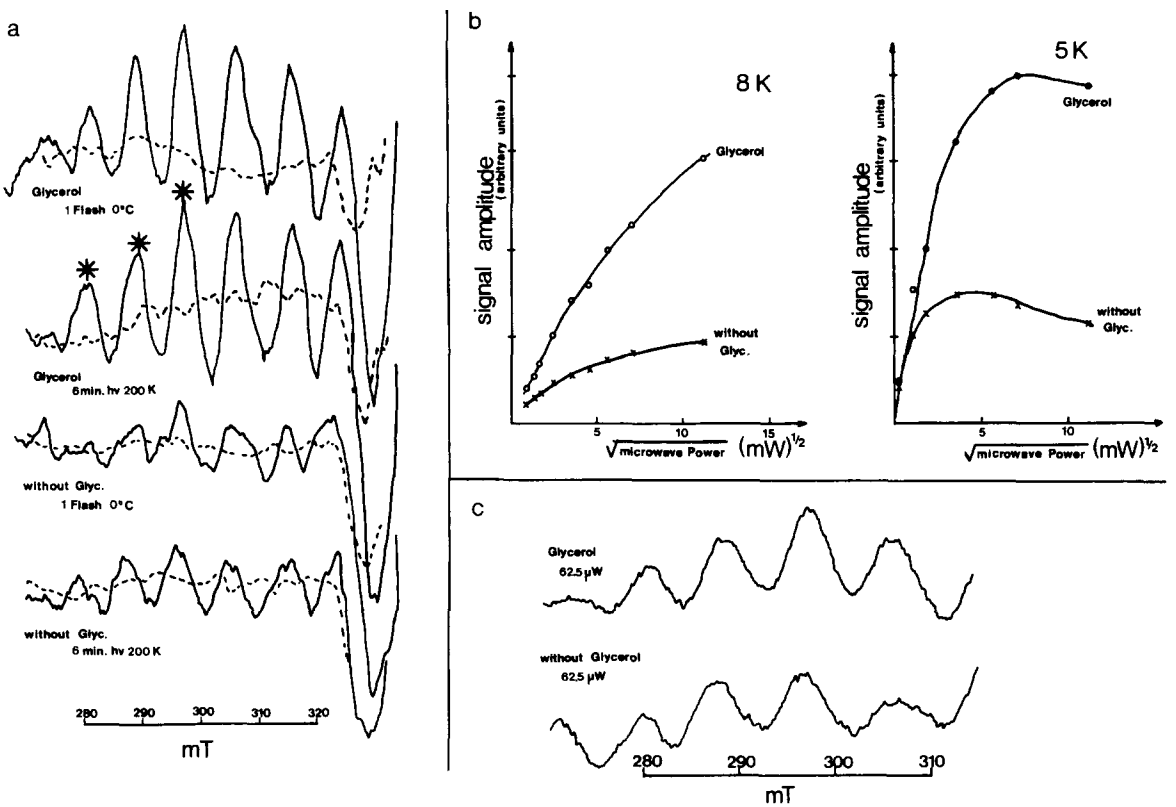

Fig. 3. Effect of glycerol on the saturation of the multiline signal. Frequency, 9.45 GHz. (a) Multiline signal photoinduced by one flash at 0 °C or by continuous illumination at 200 K. Temperature, 4.8 K; microwave power, 31.5 mW; modulation amplitude, 20 G. (b) Saturation curves of the signal photoinduced by illumination for 6 min at 200 K in the presence or absence of glycerol. Modulation amplitude, 25 G. The amplitude of the signal was measured as the height of the three downfield peaks marked *. (c) Multiline signal photoinduced by continuous light at 200 K in the presence or absence of glycerol. Instrument settings: temperature, 8 K; microwave power, 62.5 μW; modulation amplitude, 25 G. Average of eight spectra.

glycerol has a strong effect on the saturation properties of the multiline signal; this effect can be explained by changes in the relaxation properties of the species, possibly due to interactions between glycerol and manganese through hydrogen bonds. However, our measurements cannot completely exclude that glycerol also results in a slight increase in centres exhibiting the multiline signal, possibly due to increased stability of S_2 in the presence of glycerol.

Deactivation experiments

The stability of the species giving rise to the multiline signal has been studied by measuring its size in samples given one saturating flash at room temperature followed by freezing after a variable dark time. Fig. 4a shows two phases of deactivation, one of which is extremely slow. When these samples are illuminated further with continuous light at 200 K, 100% of the signal is reinduced, indicating that deactivation back to S_1 has occurred.

When samples of PS II membranes were given two flashes at 20 °C and left in darkness at this temperature for various times before freezing, the size of the multiline signal decreases, as shown in Fig. 4b. A transient increase in multiline signal which might have been expected if a shorter-lived S_3 decayed via a longer-lived S_2 could not be detected. This indicates that the S_3 state, generated by the two flashes is either more stable than S_2, or does not deactivate via S_2. This in fact agrees with the deactivation measurements done in this kind of preparation using the O_2 electrode [15] and complements the deactivation data on the multiline signal in chloroplasts already reported by Brudvig et al. [4].

Fig. 5 shows a comparison of the deactivation kinetics at 25 °C of S_2 generated by flash at 0 °C or by continuous light at 200 K. When S_2 is formed by a flash, its deactivation is slower than when accumulated at low temperature. Indeed, at 0 °C, it is thought that the electron generated on the acceptor side is transferred as far as Q_B^- and possibly as far as the exogenous acceptor, phenylbenzoquinone. Thus, recombination deactivation, which is a major deactivation route for S_2, occurs from Q_B^- [23] ($t_{1/2}$ = 25–30 s 20 °C [24]) or not at all. At 200 K on the other hand, the distance that the electron can move away from the reaction centre is limited by temperature. It is shown below that continuous illumination at 200 K results in more than one turnover of PS II taking place. Thus, it is thought that the $Q_A^- \rightarrow Q_B$ electron transfer, although slowed down at 200 K, still occurs during the course of a continuous illumination at this temperature and more than one electron can be accumulated on the quinone-acceptor

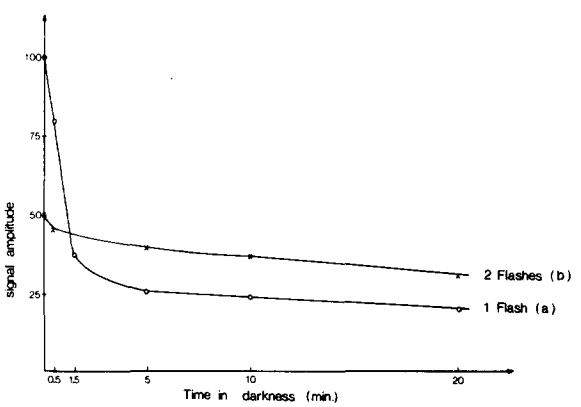

Fig. 4. Deactivation of the multiline signal. The PS II membranes were given one or two flashes at 20 °C and left to incubate at this temperature before EPR spectra were recorded. The amplitude of the signal was measured as in Fig. 3b. Instrument settings: temperature, 8 K; microwave power, 31.5 mW; frequency, 9.46 GHz; modulation amplitude, 25 G.

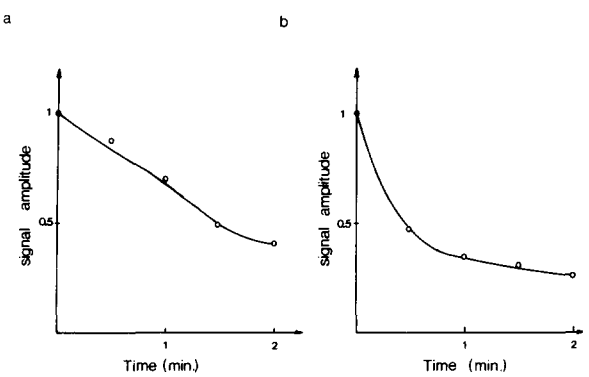

Fig. 5. Deactivation of the multiline signal photoinduced (a) by one flash at 0 °C or (b) by continuous light at 200 K. The measurements were done just after illumination and after warming for various times to 20 °C in darkness: samples at 200 K were rapidly plunged in water at 20 °C and left in darkness for the indicated time. The amplitude of the signal was measured as in Fig. 4. Instrument settings were as in Fig. 3a.

complex. These electrons can thus be involved in recombination deactivation during warming before forward electron transfer to phenylbenzoquinone takes place in all centres. This partially explains the observed behaviour although the involvement of a temperature-dependent charge stabilization event (conformational change and/or protonation) may also be considered. This possibility is under investigation.

The $g = 4.1$ signal

As already aforementioned, illumination of PS II membranes at 200 K generates both the multiline and the $g = 4.1$ signals, and the Q_A^-Fe signals. In this section, some results on this new $g = 4.1$ signal are presented.

Photochemistry

When illumination is carried out at 77 K with continuous light (not shown), or at room temperature with flash excitation (Fig. 1b), this signal is not photoinduced. Tris-washed PS II membranes illuminated at 200 K do not exhibit the signal either (Fig. 1c). These results indicate that the $g = 4.1$ signal arises from a species located on the donor side of Photosystem II, which undergoes oxidation at 200 K. That the signal cannot be trapped by one or more flashes at 0 °C suggests that it may arise from a transient species which can be trapped at 200 K. When samples are poised with DCMU (40 μM) before illumination at 200 K, the $g = 4.1$ signal is virtually absent and the multiline is slightly larger (10%) than in samples containing no DCMU (Fig. 6). These results indicate that a second turnover is necessary for the formation of the $g = 4.1$ species by illumination at 200 K and suggests that the component represents an oxidation state higher than S_2. Since the signal is not formed by two flashes at 0 °C, the signal is not S_3 itself. Thus, is seems possible that it is a component which is a 'pre-S_3' state. The slight increase of the multiline signal in the DCMU samples can be explained by interactions between this signal and the $g = 4.1$ signal.

Fig. 7a shows the effect of slow warming to 220 K on a sample previously illuminated at 200 K. At the beginning, both the multiline and the $g = 4.1$ signals are present at the maximum extent photo-

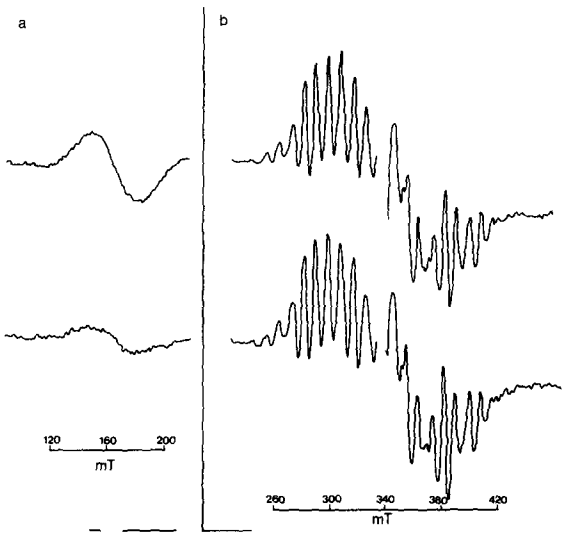

Fig. 6. Effect of DCMU upon the generation of the $g = 4.1$ (a) and S_2 (b) signals. PS II membranes ([Chl] ≈ 12 mg/ml) were incubated with 2 mM EDTA with no further addition (upper trace) or with 40 μM DCMU (lower trace) and illuminated for 4 min at 200 K. Instrument settings: temperature, 15 K; microwave power, 31.5 mW; modulation amplitude, 20 G; frequency, 9.46 GHz. Difference (light minus dark) of the average of two spectra.

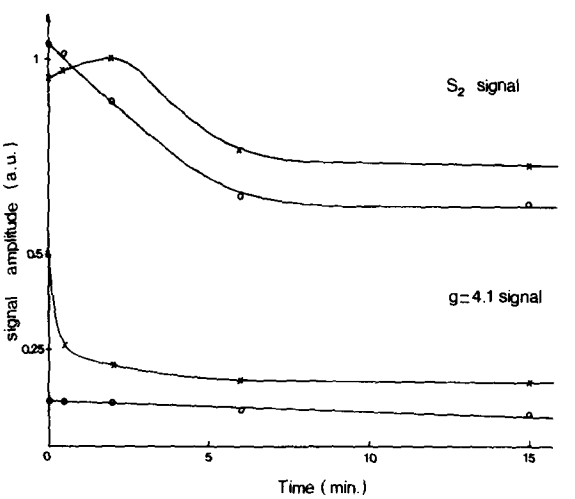

Fig. 7. Traces: a, ×——×, no additions; b, O——O, DCMU-added. Effect of warming on the multiline and $g = 4.1$ signals. Samples were illuminated for 4 min at 200 K and then warmed to 220 K and left at that temperature in darkness for various times. The amplitude of the multiline signal was measured as in Fig. 3b; that of the $g = 4.1$ signal was measured as the peak-to-peak height. Instrument settings were as in Fig. 6.

induceable at 200 K. Upon warming, the $g = 4.1$ signal decreases rapidly in the first minute, with a concomitant increase of the multiline signal. Then the two species disappear slowly.

The fast decay of the $g = 4.1$ signal in the first minute of warming indicates that deactivation of this species occurs preferentially at that temperature, suggesting that it is less stable than S_2, thus possibly located closer to the reaction centre.

This fast phase of decay of the $g = 4.1$ signal is accompanied by a slight increase (5%) of the multiline signal. This increase probably corresponds to the increased signal size observed in the absence of the $g = 4.1$ signal in the presence of DCMU (see above).

The increase in the S_2 multiline signal as the $g = 4.1$ signal decreases results either because of an increase in S_2 concentration for photochemical reasons or an increase in signal intensity due to changes in magnetic interactions. S_2 concentration would increase under such circumstances if the $g = 4.1$ signal represented a pre-S_2 state that relaxed upon warming to form the S_2 multiline signal (see Ref. 16). However, the fact that DCMU almost completely blocks the formation of the $g = 4.1$ signal indicates that it arises from an oxidation state higher than S_2. On the other hand, S_2 concentration could be increased by deactivation of such a higher oxidation state if the $g = 4.1$ signal represented a higher oxidation state of the same component that gives rise to the multiline signal (i.e., a 'S_2^+' state). This explanation seems unlikely for the following reasons. Firstly, the size of the two signals would be expected to be inversely proportional, but we have observed only a 10% decrease in the multiline signal when the $g = 4.1$ signal is photoinduced to a maximum extent at 200 K. Secondly, a recent saturation study shows that the S_2 signal concentration is unchanged by the presence of the $g = 4.1$ signal if the measurements are made at non-saturating microwave powers (Rutherford, A.W. and Zimmermann, J.L., unpublished data). Thus, it is concluded that the multiline signal and the $g = 4.1$ signal arise from two different components, between which there is a magnetic interaction that results in a decrease in the multiline signal amplitude, when measured under these conditions of temperature and power.

The same warming experiment has been done with PS II membranes poised with DCMU (40 μM) and the results are shown in Fig. 7b. As mentioned above, the multiline signal is slightly larger than without DCMU just after illumination and the $g = 4.1$ signal is very small (20% of that without DCMU). No transient increase in the multiline signal is visible in the first minute of warming. According to the discussion above, this seems to be due to the absence of interaction with the $g = 4.1$ signal.

The decrease in the multiline signal in this experiment, with and without DCMU, is unexpected, since S_2 deactivation by recombination measured by other techniques does not apparently occur at temperatures below approx. 0 °C [23]. Further experiments are required before the mechanism involved in this low temperature deactivation which occurs in some of the centres can be understood.

Spectral properties of the $g = 4.1$ signal

The signal has a g value of 4.1 and a peak-to-peak width of approx. 360 G. No hyperfine structure was visible even when observed with 2 G modulation amplitude (not shown). The possibility that this signal is due to the $\Delta m = 2$ transitions of the species giving rise to the multiline signal seems unlikely, since the two signals apparently arise from different components (see the preceeding subsection Photochemistry). The $g = 4.1$ signal appears on top of the narrow signal at $g = 4.3$, which does not seem to have any functional role in PS II photochemistry, and which is thought to be due to rhombic ferric iron, perhaps in some kind of storage form. It is tempting to invoke the existence of a functional iron in the oxygen-evolving enzyme (see Ref. 16). However, it is of note that manganese polynuclear clusters can exhibit structureless EPR spectra in that region (Refs. 25 and 26; Dismukes, G.C., personal communication).

Concluding remarks

The data reported here confirm the assignment of the EPR multiline signal as arising from S_2 and demonstrate that $Q_A^- \rightarrow Q_B$ electron transfer probably occurs even at 200 K.

The results on the new EPR signal at $g = 4.1$

suggest that it arises from an intermediate state between S_2 and S_3. If the signal observed here and suggested to be a pre-S_3 state is the same as that reported by Casey and Sauer [16] and thought to be a pre-S_2 state, this indicates that the $g = 4.1$ component acts as an electron carrier between the S states and the reaction centre, perhaps as a donor to the component which gives rise to Signal II_{vf} [27,28].

Acknowledgements

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