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Water binding to the oxygen-evolving system of chloroplasts; effects of isotope substitution on the S_2 state EPR spectrum

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Replacement of H_2O by ^2H_2O in oxygen-evolving Photosystem II preparations caused an increased resolution of the fine structure of the S_2 state EPR spectrum. In both ^2H_2O and H_2O samples, comparison of the S_2 spectra generated by illumination at 200 and 283 K (10 °C) showed a difference in the fine structure on the hyperfine lines. A reduction in the spacing of the outer hyperfine lines was also observed when samples illuminated at 283 K were compared to those where S_2 was formed by 200 K illumination. The observations are interpreted as due to proton binding, perhaps as water, at or near the manganese complex giving rise to the S_2 signal.

Introduction

Oxidation of water by Photosystem II (PS II) involves the sequential accumulation of four oxidising equivalents by the oxygen-evolving complex. Therefore, the oxygen-evolving complex cycles through five oxidation states, S_0–S_4 [1], electrons being removed by single turnovers of the PS II reaction centre. The S_0 and S_1 states are relatively stable in the dark, with the S_0 state being slowly oxidised to S_1 during dark adaptation [2]. The intermediate states, S_2 and S_3, are rapidly reduced to lower S states in the dark by reducing equivalents from the PS II electron acceptors. Oxygen is released during the conversion of the unstable S_4 to S_0. Electron paramagnetic resonance (EPR) studies have shown that the S_2 state exhibits a multiline EPR signal characteristic of manganese ions in a mixed valence binuclear or tetranuclear complex [3–10]. The manganese complex is probably the site of the accumulation of oxidising equivalents. Recent evidence has also suggested that manganese may be the site of water binding [11,12]. Hansson et al. [11] studied the effect of ^{17}O-labelled water on the S_2 multiline signal. Line broadening was induced by the ^{17}O nucleus ($I = \frac{5}{2}$), showing that oxygen ligands from water are probably associated with manganese in the S_2 state. However, under the conditions used (200 K illumination to convert S_1 to S_2) water could have bound in the S_0, S_1 or, perhaps, S_2 state. Beck et al. [12] used a water analogue, ammonia, to change the characteristics of the S_2 multiline signal formed at room temperature. When S_2 was formed by illumination at 200 K, the presence of ammonia did not change the signal. This indicated that ligand exchange only occurred at room temperature following formation of S_2. From these studies, it was suggested that the nor-

Abbreviations: EPR, electron paramagnetic resonance; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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mal substrate, water, binds to the manganese complex during turnover of the oxygen-evolving complex. These ideas were taken further in a model for water oxidation put forward by Brudvig and Crabtree [13]. However, the results of Radmer and Ollinger [14], using oxygen isotopes and mass spectrometry, showed that if water binding occurred during S state turnover, it must be able to exchange with bulk water. In this paper the binding of water to the oxygen-evolving complex has been examined by comparing the normal multiline S_2 EPR signal with that obtained when water was replaced by ^2H_2O. Changes in the spectral resolution and characteristics of the signal indicate that protons bind at or near the manganese complex.

Experimental procedure

Photosystem II was prepared by the method of Ford and Evans [15] from market spinach (*Spinacia oleracea*) or greenhouse-grown pea (*Pisum sativum*, var. Feltham First). It was resuspended and stored at 77 K in 20 mM Mes/5 mM $MgCl_2$/15 mM NaCl/20% glycerol (buffer A). Preparations with oxygen evolution rates of 400–1000 μmol O_2/mg Chl per h were used.

Samples were treated as follows: (a) The PS II preparation was resuspended in 50 mM Hepes buffer (pH 7.5) containing 1 mM EDTA to remove free manganese. This was centrifuged at $35\,000 \times g$ for 30 min and the resulting PS II pellet was resuspended in 50 mM Hepes buffer/5 mM $MgCl_2$/15 mM KCl/20% glycerol (pH 7.5) (buffer B) and divided into two samples. (b) These were centrifuged as above and one, termed H PS II resuspended in buffer B and the other, termed ^2H PS II, in buffer B made with ^2H_2O. (c) The centrifugation procedure in (b) was repeated and the ^2H PS II and H PS II preparations kept at approximately 8° C for 5 h in the dark (^2H_2O solutions freeze if kept on ice). (d) The procedure in (b) was repeated and both preparations kept either overnight (15 h) or over the weekend (63 h) at 8° C. (e) Each preparation was divided into two and the four tubes centrifuged as in (b). Four types of sample (1) H PS II in buffer B, (2) ^2H PS II in ^2H_2O buffer B, (3) H PS II in buffer A, (4) ^2H PS II in ^2H_2O buffer A were made. Each preparation was illuminated for 10 s, then centrifuged and resuspended again in their final buffer.

EPR spectrometry was performed at cryogenic temperatures using a Jeol X-band spectrometer with 100 kHz field modulation and an Oxford Instruments liquid helium cryostat. 0.3-ml samples in 3 mm diameter calibrated quartz tubes were used. Chlorophyll concentrations of samples and EPR conditions are described in the text.

Samples were prepared as follows: the H PS II or ^2H PS II preparations were placed in EPR tubes and dark-adapted for 4 h. This treatment produces a uniform S_1 state in the oxygen-evolving complex [2] which is in the resting state designated in Ref. 9. DCMU to a final concentration of 100 μM (1% ethanol) was added in darkness, and after 20 min the samples were either frozen in the dark or illuminated for 1 min with a 1000 W light source and frozen under illumination. Duplicate samples of each treatment were made. Freezing under illumination in the presence of DCMU produces S_2 in high yield in the dark-adapted samples. S_2 was also produced by illumination at 200 K of dark-adapted samples with a 1000 W projector for 2 min using an ethanol/solid CO_2 bath. For N^2H_4Cl treatment, ^2H PS II samples at p^2H 7.5 were used. Following dark adaptation and addition of DCMU as above, N^2H_4Cl (Sigma) was added to 80 mM and after 10 min the samples were frozen under illumination.

Results

Fig.1A shows the S_2 multiline signal obtained by freezing dark-adapted H PS II under illumination at 10° C. At $g = 2$ the radical of the electron donor termed signal II [16] is not fully shown. In the high field region below $g = 2$, the multiline signal is superimposed on the signal from the semiquinone-iron primary electron acceptor of PS II, Q [16,17].

The multiline spectrum consists of a series of hyperfine lines with average spacing of 8.75 millitesla (mT) [12]. The region at low field above $g = 2$ has previously been used to study hyperfine structure in order to avoid interference by EPR signals from free manganese or the semiquinone-iron acceptor. However, it was observed in these studies that differences between H PS II and ^2H PS II were more marked in the high field region

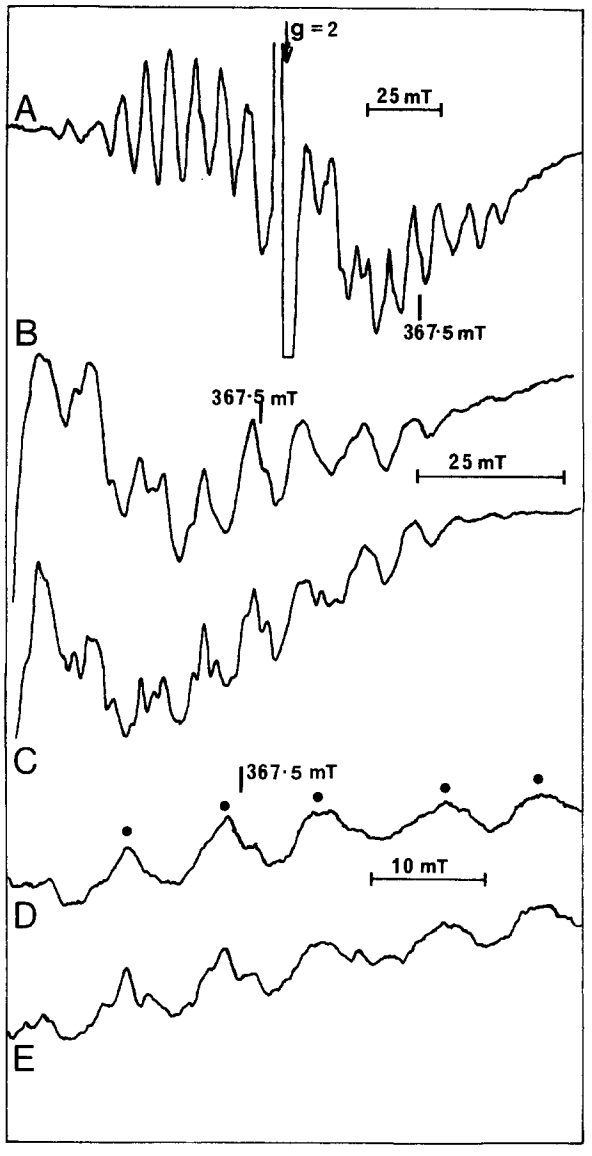

Fig. 1. Comparison of S_2 state spectra produced by illumination of pea PS II at pH 7.5. PS II-membranes (10 mg Chl/ml) were treated, as described in Experimental procedure, with 100 μM DCMU prior to illumination for 1 min at 10 °C. Samples were then rapidly frozen to 77 K. (A) 200 mT scan showing the S_2 multiline signal from H PS II. The $g = 2$ region is arrowed. (B) 100 mT scan showing the high field of the spectrum in (A) centred at 367.5 mT. (C) As (B) but ^{2}H PS II. (D) 50 mT scan of sample in (B). ●, Principal hyperfine lines. (E) 50 mT scan of sample in (C). EPR conditions: microwave frequency, 9.05 GHz; power, 5 mW (A–C) or 1 mW (D, E); modulation amplitude, 1 mT (A–C) or 0.5 mT (D, E); temperature 7 K (A–C) or 1 0 K (D, E).

below $g = 2$. Fig. 1B and C compare the high field region of the multiline spectrum of pea H PS II and ^{2}H PS II. Under conditions where both the microwave power and modulation amplitude were lowered the ^{2}H PS II multiline signal revealed more fine structure than did the H PS II preparation. Fig. 1D and E show the region centred on the field position 367.5 mT, confirming the greater spectra resolution in the ^{2}H PS II sample. This region is at higher field than any interference

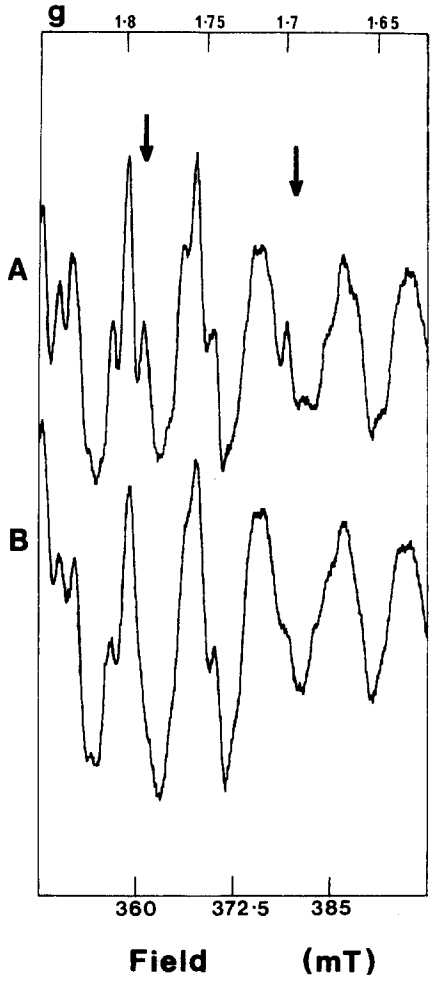

Fig. 2. High field S_2 state spectra produced by illumination at 10 °C. Comparison of pea H PS II and ^{2}H PS II at pH 7.5. PS II membranes were treated as described in Fig. 1 and Experimental-procedure. (A) ^{2}H PS II, (B) H PS II. EPR conditions as in Fig. 1, but: modulation amplitude, 0.8 mT; power, 5 mW; gain, 2000 (A) or 2500 (B); and temperature, 7.5 K. The baseline slope was subtracted to give horizontal spectra. Points where the resolution of fine structure is greatest are arrowed.

expected from either the free manganese or semiquinone-iron signals, which were of similar magnitude in both samples.

Fig. 2 compares the fine structure of the hyperfine lines in H PS II and ^{2}H PS II samples in the high field region. The S_2 signal was formed by room temperature illumination. The field positions of the maxima and minima of each principal hyperfine peak are almost identical, and so the spacing of the hyperfine lines is unchanged. However, the fine structure caused by the splitting of the principal hyperfine lines was more clearly seen in the ^2H_2O sample (arrowed). This difference was observed in both pea and spinach PS II and at both pH 6.3 and 7.5 under low microwave power and modulation amplitude conditions.

Fig. 3 compares the 367.5 mT region of S_2 multiline spectra formed by room temperature (dotted line) and 200 K illumination (solid line) in both ^{2}H PS II and H PS II samples. In samples frozen under illumination, a slightly smaller hyperfine spacing was consistently observed in both spinach and pea samples at both pH values. This shift of hyperfine peaks was most easily observed in both the high and low field extremes of the spectrum. The difference between the field positions of the 367.5 mT hyperfine line for example

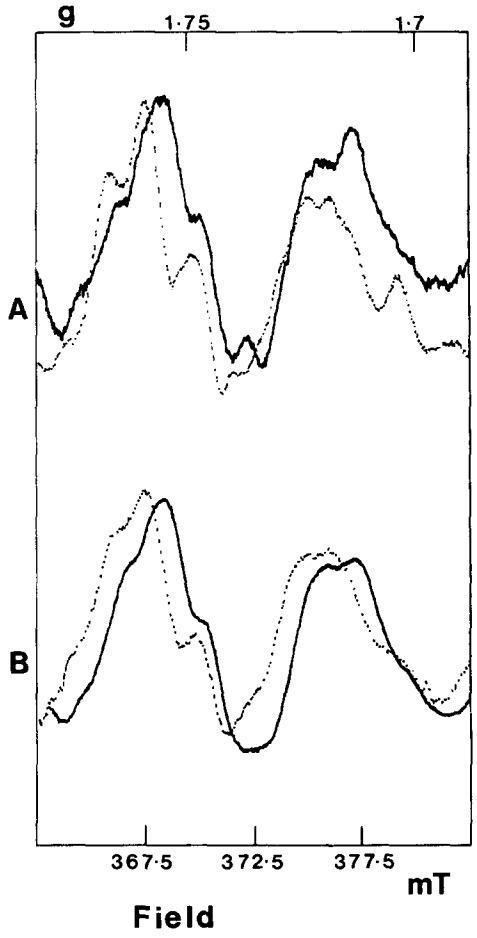

Fig. 3. Comparison of high field S_2 state spectra in spinach PS II membranes at pH 6.3. PS II membranes (8 mg Chl/mg) were treated as described in Fig. 1 and Experimental procedure. (A) ^{2}H PS II, (B) H PS II. Solid line, frozen in the dark and illuminated at 200 K for 2 min; broken line frozen under illumination after 1 min illumination at 10 °C. Conditions as in Fig. 2.

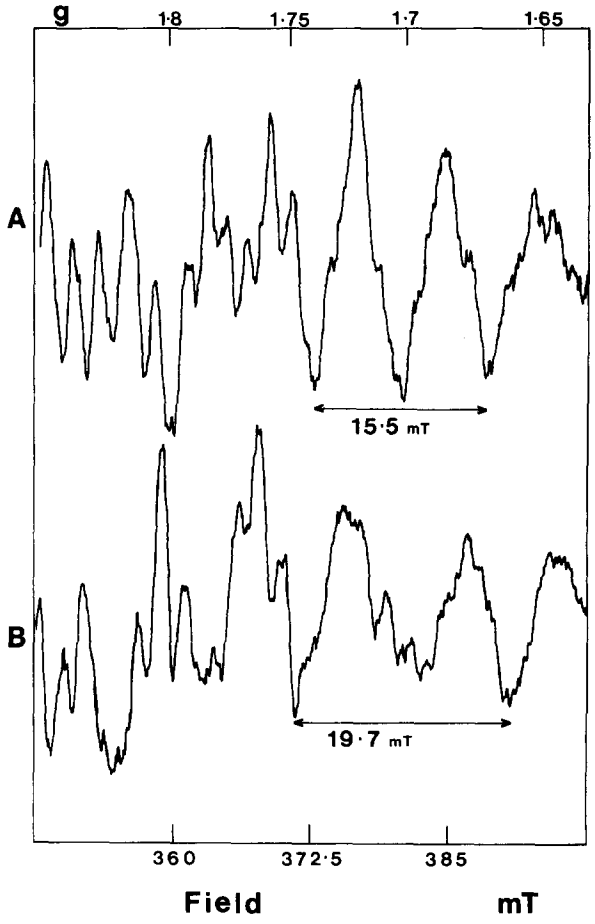

Fig. 4. Comparison of the high field S_2 state spectra of pea ^{2}H PS II with and without addition of N^2H_4Cl at p^{2}H 7.5. S_2 formed by illumination at 10 °C as described in Fig. 1. (A) With 80 mM N^2H_4Cl in ^2H_2O buffer. (B) In ^2H_2O buffer alone. EPR conditions as in Fig. 1, but: modulation amplitude, 0.4 mT; power, 1 mW; temperature, 7 K.

was 0.6–0.9 mT. The spectrum in Fig. 3 also suggests that a change in resolution of the fine structure peaks occurs between samples illuminated at different temperatures. This appears to result from a variation in the downfield shift of individual lines of the fine structure. The trough at $g = 1.7$ (arrowed in Fig. 2) was also a noticeably narrower and sharper feature in samples illuminated at 200 K, because of these shifts.

Fig. 4 compares the fine structure of the S_2 signals from ^2H PS II, both with and without addition of deuterated ammonium chloride, N^2H_4Cl. The spacing of the hyperfine lines decreases by about 20% in the N^2H_4Cl-containing sample, as found in Ref. 12 using NH_4Cl. The change in overall hyperfine pattern could result from a change in the amplitude of the individual lines of the fine structure to give the reduction in spacing observed. It was also observed that the distribution of the intensity of the hyperfine lines was different in the ammonia S_2 spectrum, with lines above $g = 2$ being relatively more intense than in the normal S_2 spectrum.

Discussion

Examination of the fine structure of the S_2 state signal confirms the observations [4,7,10] that it is composed of a multiplet of partially resolved lines arising from the manganese complex. Investigation of this fine structure has given information on the manganese ligands.

The ligand superhyperfine splittings are not fully resolved as the splitting constants are of the same order as, or less than, the intrinsic linewidths. However, the unresolved superhyperfine splitting contributes an inhomogeneous broadening to the spectrum and variations of this have been studied using isotopes of possible ligands.

Yachandra et al. [10] concluded that the fine structure was not influenced by either chloride or proton ligands to the manganese complex. In contrast, the changes in the S_2 signal seen in the present study do show an influence of protons and indicate binding of protons close to the manganese complex, perhaps as water. These broadening effects of protons overlay the fine structure which is observed in both H PS II and ^2H PS II and probably originates from the manganese cluster itself. The results also suggest that the increased resolution caused by deuterium exchange is more easily seen in samples illuminated at 10 °C (283 K) than those illuminated at 200 K (Fig. 3). In Ref. 10 the S_2 spectrum was obtained by illumination of 1-h dark-adapted samples at 190 K. This may account for the results in Ref. 10 and reflect an increased influence of protons where the S_2 state is formed before freezing. Water binding was also studied in Ref. 11, using exchange of $H_2{}^{16}O$ for $H_2{}^{17}O$. In Ref. 11, the broadening of the hyperfine lines of the S_2 signal formed by illumination at 200 K also suggested binding of water close to the manganese complex. Water appeared to be bound by the S_1 state, as binding to the S_2 state should not occur at 200 K. It is possible that the changes reflect binding of HCO_3^- but the role for this ion in PS II appears to be on the electron acceptor side. The observations in the present study suggest a difference between S_2 formed at 10 °C (283 K) and 200 K, which could reflect a change in the binding of water or protons at or near the manganese complex on formation of S_2.

In Refs. 9, 18 and 19 it was observed that several types of signal arise from the S_2 state, depending on the conditions under which it is formed. A conformational change of the manganese complex was postulated to convert short-term dark-adapted 'active' complex to 'resting' complex in long-term dark-adapted samples. The results of Forster et al. [20] suggested that the resting state was less protonated than the active state. In the conditions used in the present study, the 200 K-illuminated samples represent the resting state and the samples illuminated at 10 °C represent the active state. Therefore, the observation of the influence of protons on the active state is consistent with the current models [9,13,19] for different configurations of the complex for each of the two states.

Computer simulations of the EPR spectra from exchange-coupled Mn(III)/Mn(IV) dimeric and tetrameric complexes show striking similarities to the S_2 spectrum. These simulations [5,19] suggest that there would be least superposition of hyperfine lines in the high field region used in this study. This would lead to greater fine structure in this region as observed here.

The current models of the manganese complex also account for the reduction of spacing between the hyperfine lines on binding of ammonia to the S_2 state [12,19]. A hypothesis for two ammonia (and therefore water) binding sites near the manganese complex was proposed, one binding on S_1 formation and the other on formation of S_2 [21]. Only the (ammonia) binding on S_2 formation affected the multiline EPR signal from the manganese complex. This idea is supported by the results from both the present study and Ref. 11, using isotopes of water.

The model discussed by De Paula et al. [19] introduces the need for *g* anisotropy into computer simulations in order to reproduce the more accurately S_2 spectra. Rutherford [8] showed that the outer field lines at low field exhibited orientation dependence. The orientation dependence resulting from anisotropy would affect the outer hyperfine lines to a greater extent. Therefore, the shifts in the spacings of the hyperfine peaks on the binding of water and ammonia probably result from conformational changes in the complex which alter zero field splitting parameters. This would also account for the observed changes in the intensity of hyperfine peaks in different parts of the spectrum.

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