Investigation of the interaction of the water oxidising manganese complex of photosystem II with the aqueous solvent environment

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Abstract Interaction of the water oxidising manganese complex of photosystem II with the aqueous environment has been investigated using electron paramagnetic resonance spectroscopy and electron spin echo envelope modulation spectroscopy to detect interaction of [^{2}H]methanol with the complex in the S_2 state. The experiments show that the classical S_2 multiline signal is associated with a manganese environment which is not exposed to the aqueous medium. An electron paramagnetic resonance spectroscopy signal, also induced by 200 K illumination, showing ^{2}H modulation by methanol in the medium and a modified multiline electron paramagnetic resonance spectroscopy signal formed in parallel to it, are suggested to be associated with a second manganese environment exposed to the medium.

Key words: Photosystem II; Water oxidation; Manganese complex; Electron spin echo envelope modulation; Photosynthesis; Photosynthetic oxygen evolution

1. Introduction

The photosynthetic oxidation of water and release of oxygen is catalysed by a membrane protein complex, photosystem II, containing an essential manganese (Mn) complex [1–3]. The oxidation of water is a four electron process, while each turnover of the photosystem II reaction centre results in a one electron oxidation. A major function of the water oxidising complex is the accumulation of four oxidising equivalents prior to the release of oxygen. If water oxidising preparations are exposed to short flashes of light causing a single turnover of the reaction centre, oxygen is evolved on the third flash and subsequently after each fourth flash. This process is well-defined by the 'S' state hypothesis [4,5]. The complex has five oxidation states defined as S_0–S_4. The resting state in the dark is S_1. Each turnover of the reaction centre advances the oxidation state between S_0 and S_4. The release of oxygen, and presumably oxidation of water, occurs rapidly in the dark with the conversion of S_4 back to S_0.

Photosystem II contains a Mn complex with four Mn atoms in two distinct μ-oxo-bridged pairs. It is now clear that this Mn complex acts as the charge accumulator undergoing oxidation state changes during at least three of the S state changes. Electron paramagnetic resonance spectroscopy (EPR) and X-ray absorption spectroscopy clearly indicate different Mn oxidation states in S_0, S_1 and S_2. The evidence for Mn oxidation on the S_2 to S_3 transition is controversial, with conflicting reports from different X-ray absorption spectroscopy and EPR experiments but the central redox role of the Mn complex is clear.

The mechanism of catalysis of water oxidation is not known and the site of water oxidation has not been identified. Evidence for the role of Mn as the redox accumulator has suggested that it may also be the site of water oxidation. However, there is little direct evidence for water binding to the Mn complex.

One possible technique to detect interaction of water with the Mn complex is to use EPR to look for magnetic interactions between the complexes giving rise to the S_2 or S_0 EPR signals and water isotopically labelled either with ^{2}H or ^{17}O. Andreasson [6] reported small shifts in the S_2 multiline spectrum in the presence of H_2 ^{17}O. Nugent [7] reported some very small changes in ^2H_2O, but the latter may be re-interpreted as reflecting the overall exchange of deuterium into the protein complex. CW EPR is a relatively insensitive technique for the detection of interactions of this sort. The pulsed EPR technique of electron spin echo envelope modulation (ESEEM) offers the possibility of more specific identification of such interactions. We used ESEEM to investigate the interaction of photosystem II in the S_1 and S_2 states with either ^2H_2O or H_2 ^{17}O but were unable to detect any interaction which could be attributed to either specific binding of water to the Mn complex or to interaction between the complex giving rise to the S_2 signal and the aqueous environment [8].

The EPR signals from the Mn complex are very sensitive to the properties of the environment, particularly to the presence of alcohols. The intensity of the S_2 multiline signal is increased by the addition of alcohols, the S_0 multiline signal is only observed in the presence of methanol [9,10]. These observations suggest that there may be a specific interaction of methanol with the Mn complex and that investigation of this interaction may provide information both about the exposure of the Mn complex to the environment and possible substrate binding sites on the Mn complex.

We have now used ESEEM spectroscopy to investigate the interaction of methanol with the S_2 state of the Mn complex.

2. Materials and methods

Oxygen evolving photosystem II subchloroplast membranes were prepared from market spinach by the method of Ford and Evans [11]. Samples were prepared as in [8] except that 0.5 mM *p*-phenyl benzoquinone replaced di-methyl benzoquinone and 1% methanol, either per-deuterated or protonated, was added. The samples were converted to the S_2 state by continuous illumination at 200 K in an ethanol/solid CO_2 bath for 2–10 min. Varying the illumination periods slightly altered the concentration of the S_2 state but did not qualitatively affect the results of the experiments. EPR analysis of the prep-

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Abbreviations: EPR, electron paramagnetic resonance spectroscopy; ESEEM, electron spin echo envelope modulation

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arations used showed that no photo-reducible iron sulfur centres could be detected indicating that they are free of photosystem I.

CW EPR spectra were recorded using a JEOL RE1X spectrometer with an Oxford Instruments liquid helium cryostat. ESEEM spectra were recorded on a Bruker ESP380E X-band pulsed spectrometer equipped with a Bruker 1052 DLQ-H8907 variable Q dielectric resonator and an Oxford Instruments CF395 cryostat. ESEEM spectra were recorded as in Turconi et al. [8]. Data analysis was performed using Bruker WINEPR software. Measurements were routinely made using $\tau = 128$ ns. Preliminary experiments using two other values of τ did not show any effect of τ on detection of the ^2H modulation.

3. Results

We have measured three pulse ESEEM spectra of photosystem II preparations in the S_1, dark adapted state, and the S_2 state induced by 200 K illumination in the presence of protonated or deuterated methanol. These spectra clearly show that modulation due to ^2H can be observed in the spectrum of the S_2 state in the presence of [^2H]methanol. The modulation is seen as a peak in the FT spectra around 2.2 MHz, the frequency varies with the magnetic field essentially as the Larmor frequency for ^2H. No modulation is observed in the S_1 state. Spectra obtained across the full absorption range of the S_2 signal show ^2H modulation over a wide spectral range of about 50 mT on either side of $g = 2$ (Fig. 1), but modulation is not observed in the wings of the S_2 multiline spectrum. The spectra shown are [^2H/^1H]methanol difference spectra. In our previous experiments using $H_2^{17}O$, modulation was seen in both S_1 and S_2 states and only in part of the spectrum around $g = 1.92$. The results with methanol are clearly different showing interaction of the methanol with a light-induced spectral component. The spectral range over which modulation is observed appears to be less than the full width of the S_2 signal. The signal intensity, and so the signal to noise ratio, is however poor in the wings of the spectrum.

The multiline EPR spectrum induced by 200 K illumination in methanol containing samples is very stable during 77 K storage with little loss of the intensity over periods of months.

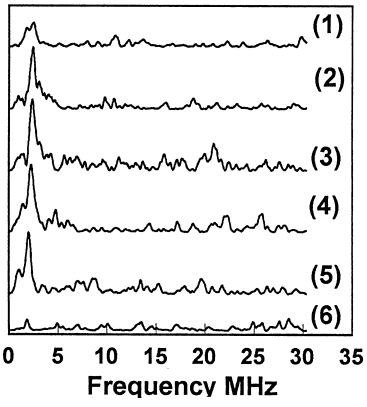

Fig. 1. Field dependence of ^2H modulation of the EPR spectrum of photosystem II after 200 K illumination in the presence of methanol. Three pulse ESEEM spectra of photosystem II samples in the presence of [^2H]methanol or ^1H-methanol were divided in the time domain and the result was Fourier-transformed to provide ^2H/^1H difference spectra. Samples used in the experiment shown were re-illuminated on two occasions, each following 3 weeks storage at 77 K. Spectra were recorded as described in Section 2 at different field positions. (1) 400 mT, (2) 380 mT, (3) 358.5 mT, (4) 337.5 mT, (5) 320 mT, (6) 300 mT (frequency = 9.70 GHz, $g = 2.00$ is at 346.5 mT)

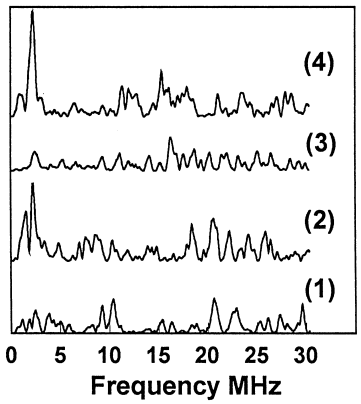

Fig. 2. The effect of 200 K illumination and 77 K storage on the observed ^2H modulation of the EPR spectrum of photosystem II in the presence of methanol. Three pulse ESEEM spectra of photosystem II in the presence of [^2H]methanol or ^1H-methanol were divided in the time domain and the result was Fourier-transformed to provide ^2H/^1H difference spectra. Spectra were recorded as described in Section 2 at 337.5 mT. (1) Dark adapted sample. (2) Sample illuminated at 200 K for the first time. (3) Sample after storage at 77 K for 4 weeks. (4) Sample re-illuminated at 200 K immediately prior to measurement.

If the ^2H modulation observed in the S_2 state samples reflects interaction of methanol with the Mn complex, it might be expected to show the same stability. However, we have found that this is not the case. No modulation is observed in samples which have been stored above liquid nitrogen for periods of 3–4 weeks, although these retain the multiline signal at only slightly less than the original intensity. The modulation is restored by re-illumination at 200 K (Fig. 2) and lost again on subsequent storage. Modulation is not restored by re-illumination at 77 K or by incubation in the dark at 200 K. Modulation is not seen if samples in the S_1 state are illuminated at 77 K. Repeated illumination at 200 K over a period of time results in an increased apparent modulation intensity.

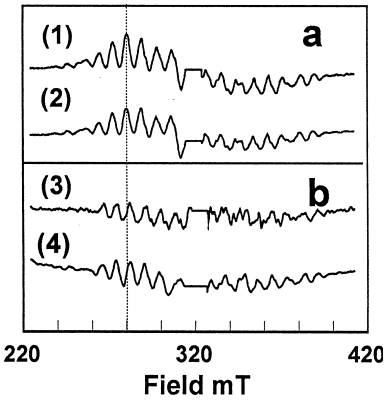

Fig. 3. EPR spectra of photosystem II in the presence of methanol. (a) Spectra of samples previously illuminated on three separate occasions and then stored at 77 K for 6 weeks. (1)Sample containing H-methanol. (2) Sample containing [^2H]methanol. (b) Samples were then illuminated at 200 K for 10 min. Spectra were again recorded and light minus dark spectra are shown. (3) Sample containing ^1H-methanol. (4) Sample containing [^2H]methanol. Spectra expanded $\times 2$ compared to (a). EPR conditions: frequency = 9.01 GHz ($g = 2.0$ is at 322.5mT); power 10 mW; modulation amplitude 1.6 mT; temperature 8 K.

Comparison of CW EPR spectra of samples containing methanol freshly illuminated at 200 K and then stored for a period during which the modulation decays, shows a very slight decay of the S_2 multiline signal. Following re-illumination at 200 K, there are again no major spectral changes, difference spectra do however show the appearance of a weak multiline signal. This signal shows some differences from the normal S_2 signal (Fig. 3), with loss of the outer lines of the spectrum. Samples with ^1H or [^2H]methanol showed the same spectral features.

4. Discussion

These experiments show that modulation of the EPR spectrum by ^2H in ^2H-labelled methanol is observed in photosystem II preparations following illumination of S_1 samples at 200 K. The modulation is not associated with the classical S_2 multiline signal. These results confirm the conclusion of our $H_2^{17}O$ experiments showing that the component of the Mn complex giving rise to the classical S_2 multiline signal is not exposed to the solvent environment. The modulated signal is less stable and appears to be narrower than the S_2 multiline. The small multiline signal formed in the CW EPR spectrum upon re-illumination at 200 K is also narrower than the classical multiline. A modified multiline signal is observed following Ca depletion [12]. That spectrum is quite different from the spectrum observed in our experiments.

The requirement for illumination to induce the signal, parallel induction of the modified multiline signal and the signal giving rise to modulation in the ESEEM spectra and their similar line widths, indicate that both the signals arise from a component of the photosystem II Mn complex. The ESEEM results show a weak interaction of methanol with the Mn complex. This result indicates that the component giving rise to the modified multiline is exposed to the solvent. There is no indication of strongly coupled deuterons.

The modulation is associated with the small multiline signal formed on re-illumination. Heterogeneity of the S_2 multiline signal has been reported by Smith and Pace [13], comparing samples with and without methanol, and by Boussac [14] observing the response of the signal to infra red illumination. These results were interpreted as indicating that the S_2 environment may be heterogeneous, causing small spectral changes including loss of intensity in the wings of the spectrum. Boussac observed a decrease in the multiline intensity on infra red illumination coupled to the appearance of the $g = 4$ signal. In these experiments, we observe a small increases in the multiline intensity on re-illumination, the signals observed on re-illumination have a narrower line width than the normal S_2 multiline with no intensity in the wings of the spectrum. It seems likely that the present experiments have isolated the spectrum indicated by the previous work. The loss of structure at the edges of the spectrum might reflect broadening of the Mn hyperfine lines due to changes in structure following addition of methanol. However, the ESEEM experiments show that the broader stable S_2 multiline is not modulated by, and so not accesible to, methanol in the medium, indicating that it is unlikely that the changes occur at the Mn centre giving rise to that spectrum.

The Mn dimers in the complex have been shown to have two different orientations in the membrane [15] and to respond differently to Ca depletion [16]. Models have been proposed suggesting different functions for the two dimers [17,18]. Our results indicate that the S_2 multiline signal is associated with a Mn dimer which is inaccessible to the solvent.

The modulated EPR signal is clearly associated with a redox centre which is exposed to the medium. The field width over which the modulation is observed and the CW EPR spectrum which develops in parallel with the appearance of modulation suggest that this is a Mn centre, but in a different environment to that in the normal S_2 state and possibly in a different redox state. The conditions of the experiment in which the modulated multiline signal is repeatedly induced, with an increasing intensity, in samples which have been maintained at temperatures below 200 K suggests that the modified environment is unlikely to represent changes in the protein structure or damage to reaction centres, although that cannot be completely excluded. It may be simplest to consider that the signals are associated with two dimers in different positions in the membrane. It may reflect the transfer of the S_2 redox state from an 'inner' to an 'outer' dimer in a fraction of centres. It is not apparent why that should require illumination, although Boussac [14] has shown that illumination can affect the S_2 state by changing the spin state. It may be possible that an excited state is required for electron transfer between two dimers at 200 K. Alternatively, the signal might be associated with a change of the redox state of the Mn complex.

The S_0 state, thought to involve a dimer in the Mn^{2+}-Mn^{3+} state, has a wider multiline signal than the S_2 state, thought to formally reflect a Mn^{3+}-Mn^{4+} dimer. It is, therefore, unlikely that the narrower modulated signal is due to the S_0 state. A narrower multiline signal might be associated with a higher oxidation state, perhaps Mn^{4+}-Mn^{5+}, of the Mn complex. Extensive characterisation of the signal will be required to determine its origin.

These experiments identify two Mn multiline signals associated with two different Mn environments. They can be interpreted as confirming the association of the classical S_2 multiline with an 'inner' Mn dimer environment and a modified S_2 signal associated with an 'outer' Mn dimer environment. However, the possibility that they identify a higher oxidation state of the Mn complex with a Mn dimer in the water oxidation site should be considered. The detection of two distinct Mn environments, one of which is exposed to the aqueous environment, strengthens the hypothesis that the Mn complex is the site of water oxidation.

After the initial submission of this paper, a paper by Force et al. [18] was published, describing ESEEM studies of the interaction of small alcohols, including methanol, with the S_2 state. As in the experiments reported here, weakly coupled deuterons were observed. They conclude that the coupling arises from the non-exchangeable methyl deuterons of methanol bound to the Mn complex, with the deuterons 3.6–5 Å from the Mn. We have made the less specific conclusion that the weakly coupled deuterium ESEEM arises from exposure of a Mn site to the methyl deuterons of methanol in the environment. The observation of modulation from weakly coupled deuterons implies a distance of 4–6 Å from the Mn centre. Force et al [18] did not report the field dependence of the ESEEM or observe the decay of the ESEEM signal and the correlation with a small multiline component reported here.

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