Evidence for the presence of a component of the Mn complex of the photosystem II reaction centre which is exposed to water in the S_2 state of the water oxidation complex

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Abstract The interaction of water oxidising photosystem II preparations with the aqueous environment has been investigated using electron spin echo envelope modulation spectroscopy in the presence of ^2H_2O. The spectra show interaction of ^2H of ^2H_2O with the preparation in the S_2 state. The component interacting with water decays during 1–4 weeks storage at 77 K. No interaction of water with the classical multiline S_2 Mn signal, which is more stable on storage at 77 K, was detected. The results show that a component of the water oxidation complex, possibly involving the Mn centre, is accessible to water and may be the water binding site for photosynthetic water oxidation.

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

1. Introduction

The oxidation of water in photosynthesis is catalysed by a membrane–protein complex, photosystem II, containing an essential manganese complex [1–3]. The oxidation of water is a four electron process, while the photosystem II reaction centre turnover is a one electron process. Water oxidation involves the accumulation of oxidising equivalents by a water oxidising complex associated with each photosystem II reaction centre. Classical experiments investigating the evolution of oxygen following single turnover flash illumination show water oxidation from dark adapted samples peaks on the third flash and subsequently every fourth flash. This process is described by the 'S' state hypothesis [4,5]. The complex has five formal oxidation states S_0–S_4. The dark stable resting state is S_1. Each turnover of the reaction centre advances the oxidation state between S_0 and S_4, the oxidation of water and release of oxygen then occurs rapidly in the dark, with the return of the complex to the S_0 state.

Photosystem II contains four Mn atoms in a complex made up of two distinct μ-oxo-bridged pairs [1–3]. This Mn complex has been shown to act as the accumulator of oxidising equivalents for water oxidation undergoing oxidation state changes on at least three of the S state transitions. Electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy (XAS) have shown that the Mn oxidation state changes between S_0, S_1 and S_2. The evidence for Mn oxidation on the S_2 to S_3 transition is controversial, with conflicting results and interpretation of different XAS and EPR experiments. However, the central role of the Mn complex is clear.

The mechanism of catalysis of water oxidation is not known. Although many models have been suggested, the site of water binding and oxidation has not been identified. Characterisation of the Mn complex as the accumulator of oxidising equivalents has suggested that it may also provide the catalytic site for water oxidation. However, it has proved difficult to obtain strong evidence for interaction of water with the Mn complex.

The work of Wydrzynski and coworkers [6] indicates that water exchanges with the water oxidation complex in the S_3 state, they detect two water exchange rates, indicating the presence of two water binding sites in S_3. Characterisation of the EPR spectra of the S_0 and S_2 oxidation states offers the possibility of detecting interaction of water with these oxidation states using water labelled with either ^2H or ^{17}O and looking for magnetic interaction between these nuclei and the Mn complex giving rise to the EPR signals. Two reports of attempts to identify such interactions by CW EPR have been published. Andreasson [7] identified small shifts in the S_2 multiline spectrum in the presence of H_2 ^{17}O. Nugent [8] reported some very small changes in ^2H_2O, but the latter may be reinterpreted as reflecting the overall exchange of deuterium into the proteins of the complex. The pulsed EPR technique of electron spin echo envelope modulation (ESEEM) offers a much more specific and sensitive technique to detect either specific binding of magnetic nuclei to redox centres with EPR signals or less specific contact with nuclei in the immediate environment of the redox centre. We have previously reported the use of ESEEM spectroscopy to investigate the interaction of H_2 ^{17}O and ^2H_2O with the S_1 and S_2 states of the Mn complex [9]. We were unable to detect any specific interaction which could be assigned to interaction of the Mn complex with water.

The EPR spectra of the Mn complex are sensitive to the presence of alcohols in the medium, the strongest effects are observed with methanol. Methanol is required to observe the multiline spectrum from the S_0 state [10]. In the S_2 state two EPR signals are observed in the absence of alcohols, one around $g=4$ and one a multiline signal around $g=2$. Methanol removes the signal observed in the $g=4$ region convert-

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Abbreviations: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; XAS, X-ray absorption spectroscopy

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ing it to the multiline signal. Recently two papers have reported detection of interaction of methanol with the S_2 state using ESEEM spectroscopy. Force et al. [11] observed weak interaction of the deuterons from methyl deuterated methanol calculating a distance from the Mn centre of 3.6–5 Å. They suggested the methanol was bound to the Mn through the hydroxyl group. In similar experiments we observed weak interaction, essentially at the Larmor frequency of deuterium compatible with a Mn deuterium distance of 4–6 Å [12]. However, we found that the component interacting with the ^2H was metastable, decaying over a period of weeks during storage at 77 K. This is in contrast to the behaviour of the classical component giving rise to the S_2 multiline signal in the presence of methanol which is stable for very long periods at 77 K. Samples still show strong multiline Mn signals after up to 2 years storage. The ESEEM signal also showed a narrower field range than the classical multiline signal. Parallel CW EPR experiments suggested that the ESEEM signal may be associated with a second Mn component with a slightly different multiline signal.

We have now repeated our experiments with samples prepared in ^2H_2O in the light of our results obtained with methanol indicating the presence of a metastable component accessible to the aqueous medium.

2. Materials and methods

Oxygen evolving photosystem II subchloroplast membranes were prepared from laboratory grown pea seedlings by a method based on that of Ford and Evans [13]. Oxygen evolution rates were in the range of 350–450 μmol/mg chlorophyll/h. Preparations were transferred into ^2H_2O by pelleting the preparation from the protonated medium and resuspending into medium containing 5 mM $MgCl_2$, 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 15 mM NaCl, 0.33 M sucrose prepared in 99.9% ^2H_2O adjusted to pD 6.7 with NaOH. Samples were prepared as in [9] except that 1 mM phenyl *p*-benzoquinone replaced dimethyl benzoquinone and 2 or 4 h dark adaptation was used. The samples were converted to the S_2 state by continuous illumination at 200 K in an ethanol/solid CO_2 bath for 10 min. EPR analysis of the preparations used showed that no photo-reducible iron–sulphur 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. [9]. 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. The pulse train repetition rate was 10 Hz.

3. Results

We have measured three pulse ESEEM spectra of samples in the S_1 and S_2 states in ^1H_2O and ^2H_2O. Spectra were recorded at a number of field positions within 50 mT of $g = 2.00$. Modulation of the spectrum by deuterium was observed essentially at the Larmor frequency of deuterium in the ^2H_2O samples (Fig. 1A). Dark adapted S_1 samples (Fig. 1A, curve 1) showed weak modulation which decreased with longer dark adaptation of the sample prior to freezing. After 16 h dark adaptation there was no detectable modulation although

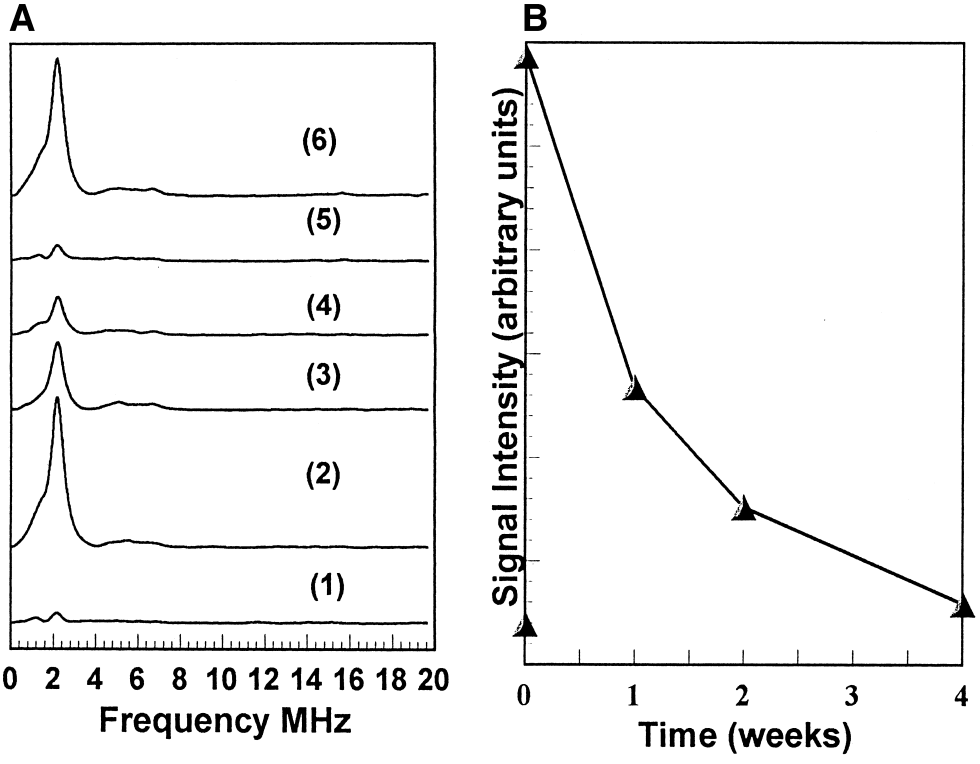

Fig. 1. A: The effect of 200 K illumination and subsequent storage at 77 K on the ESEEM spectrum of photosystem II in ^2H_2O. Three pulse ESEEM spectra were recorded as described in Section 2 at 337.5 mT. Dark adapted sample (1); after 200 K illumination (2). After storage at 77 K for 1 week (3), 2 weeks (4), 4 weeks (5). After reillumination at 200 K following 4 weeks storage at 77 K (6). B: Changes in the intensity of the ^2H modulation of the spectra shown in A with time. The intensity of the modulation was measured at 2.2 MHz. The lower symbol on the *y* axis is the initial dark intensity, the upper symbol the intensity immediately after illumination at 200 K.

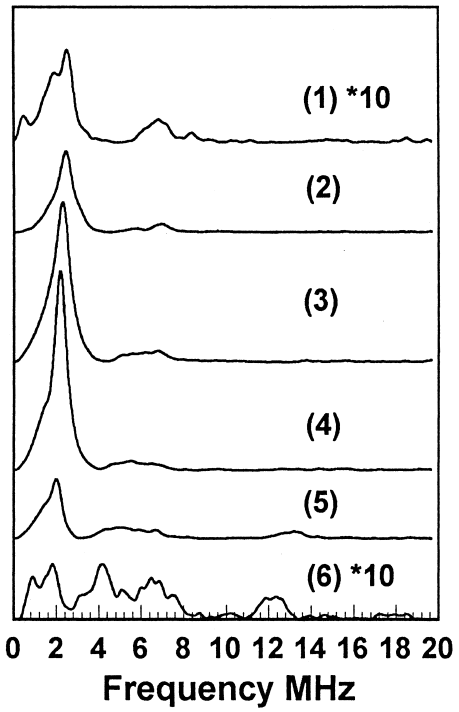

Fig. 2. Field dependence of ^2H modulation of the EPR spectrum of photosystem II in ^2H_2O after 200 K illumination. Three pulse ESEEM spectra were recorded at different field positions as described in Section 2 immediately following the illumination at 200 K of a dark adapted sample. (1) 398.5 mT. (2) 378.5 mT. (3) 358.5 mT. (4) 337.5 mT. (5) 317.5 mT. (6) 297.5 mT. Spectra 1 and 6 are expanded ×10. (Frequency = 9.71 GHz, $g = 2.00$ is at 346.9 mT.)

the samples remained fully active. Four hours dark adaptation was used for the experiments shown in the figures. Following illumination at 200 K to induce the S_2 state, very strong modulation was observed at the Larmor frequency (Fig. 1A, curve 2). The intensity in the Fourier transform of the ^2H modulation in the 4 h dark adapted sample before 200 K illumination was less than 10% of that after illumination. Subsequent storage of the sample at 77 K resulted in the decay of the modulation (Fig. 1A, curves 3–5). This decay was faster than we observed in our experiments with [^2H]methanol [12] and was complete within 4 weeks (Fig. 1B). When essentially all of the modulated signal induced by 200 K illumination (Fig. 1, curve 5) had decayed a large part of the multiline Mn signal (Fig. 3, curve 3) and $Q_A^{\bullet-}$ signal could still be observed in the sample.

The modulation could be observed at field positions to approximately 50 mT on either side of $g = 2.00$ (Fig. 2). The modulation was strongest close to $g = 2.00$ on the low field side, but was observed essentially symmetrically around $g = 2.00$. Modulation was not observed at field positions more than 50 mT from $g = 2.00$, but the overall signal intensity was very low at these field positions making the signal to noise of the ESEEM spectra very poor.

Four redox components which show major changes on 200 K illumination can be readily identified in the EPR spectra of photosystem II preparations. These are the oxidised tyrosine $Y_D^{\bullet+}$ radical, reduced $Q_A^{\bullet-}$, the multiline signal due to the S_2 state of the Mn complex and cytochrome b_{559} (data not shown). A major part of these signals is stable to storage at 77 K over the 1–4 week period used in these experiments. A slow decay thought to be due to back reaction between $Y_D^{\bullet+}$ and $Q_A^{\bullet-}$ is observed; however, a large part of these two components was unchanged at the end of the storage period. There is also some decay of the multiline signal as we reported previously for samples in the presence of methanol. We have now observed that a decay of part of the multiline signal also occurs at 77 K in samples without methanol. This decay occurs more rapidly than in the presence of methanol. Fig. 3 shows the changes in the EPR spectrum of photosystem II following 200 K illumination (Fig. 3, curve 1) and following storage at 77 K (Fig. 3, curves 2 and 3). The spectrum is dominated by the multiline S_2 signal centred at about $g = 2.00$. $Q_A^{\bullet-}$ is also present, and is centred at about $g = 1.9$ with most of the signal intensity on the high field side of $g = 2.00$. The narrow signal of $Y_D^{\bullet+}$ has been deleted from the centre of the spectrum as it is 'off scale' under these conditions. Fig. 3, curves 4 and 5 show the difference spectra between the sample immediately following 200 K illumination and after 1 and 4 weeks storage at 77 K. These experiments show that during storage at 77 K there is some decay of three of the EPR signals induced by 200 K illumination. The decay of the multiline is more pronounced than in samples containing methanol. However, even after long storage when the ^2H modulation has completely decayed a major part of the multiline signal remains, with little further change if the experiment is followed for 13 weeks. Cytochrome b_{559} does not change significantly during this storage at 77 K (data not shown).

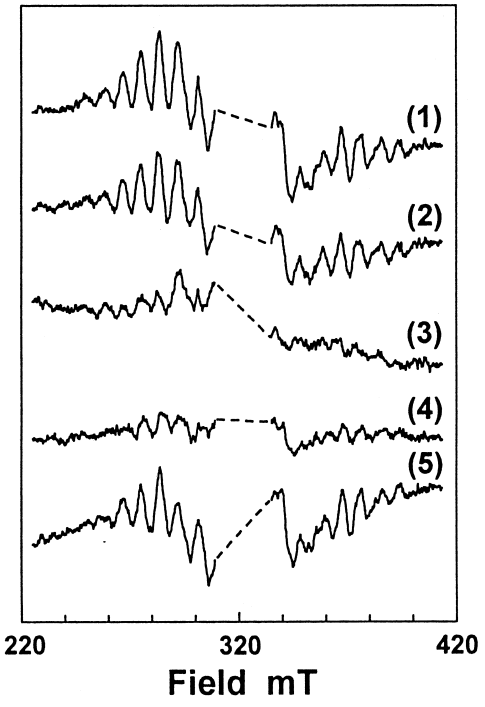

Fig. 3. EPR spectra of photosystem II showing the effect of storage at 77 K on the spectrum of the multiline signal of the Mn complex. A dark adapted sample was illuminated at 200 K and the spectrum recorded (1). The sample was then stored for 1 week at 77 K and the spectrum recorded (2) and again after 4 weeks storage (3). The spectra shown (1–3) are difference spectra in which the initial dark spectrum was subtracted from the spectra of the illuminated or stored samples. Difference spectra (4) of 1−2 and (5) of 1−3 show the spectrum of the decaying multiline component. EPR conditions: frequency 9.05 GHz ($g = 2.0$ is at 323.3 mT), power 10 mW, modulation amplitude 1.6 mT, temperature 8 K.

If the stored sample is illuminated at 77 K, oxidation of Y_D and reduction of Q_A is observed. There is no change in the multiline signal, nor in the intensity of the ^2H modulation signal on 77 K illumination.

Spectra of photosystem II preparations may show EPR signals due to $Q_B^{\bullet-}$ in the $g = 1.9$ region. As with $Q_A^{\bullet-}$ the intensity of these signals is largely on the high field side of $g = 2$. Q_B may be displaced from photosystem II by the inhibitor DCMU. Addition of 0.1 mM DCMU did not affect the appearance of the ^2H modulation following 200 K illumination, nor its subsequent decay, in these experiments.

4. Discussion

The results presented in this paper parallel those we have presented earlier demonstrating the interaction of methanol with photosystem II in the S_2 state [12]. The experiments show that a weak magnetic interaction between ^2H in water and a component of the photosystem II reaction centre can be detected by ESEEM in the S_2 state immediately after 200 K illumination. However, the interaction is not with the S_2 state as it is normally defined in that the ESEEM modulation decays over a relatively short time at 77 K when other components of the S_2 state such as the multiline Mn signal and Q_A^- are in part stable.

The results are different from those we published following an earlier investigation of the interaction of water with photosystem II [9]. The experiments differ from those described in [9] in two ways. The samples were exchanged into ^2H_2O by the simple procedure of pelleting the photosystem II particles and resuspending in ^2H_2O medium, rather than by freeze drying and resuspending in ^2H_2O. In the present experiments the ESEEM spectra were obtained immediately (within 8 h) of 200 K illumination. In the previous experiments [9] samples were illuminated, the CW EPR spectra measured to confirm the presence of the multiline signal, and then stored at 77 K before the ESEEM spectra were recorded. The presence of the multiline spectrum was again confirmed after the ESEEM spectroscopy. It is unlikely that the failure to observe the interaction was due to the freeze drying, the samples were active in a wide range of assays. Experience in the present experiments, and in the experiments with methanol, suggests that failure to observe modulation in the experiments in [9] may have been due to decay of the modulated signal between the time of the 200 K illumination and the ESEEM measurements.

In [12] we discussed the possibility that the ESEEM signal was associated with a component of the multiline Mn signal reflecting a Mn centre in a different environment to that of the major component of the signal. The same conclusion may be drawn from the present experiments. Cytochrome b_{559} is unlikely to be the component showing modulation. It is stable during storage at 77 K, and its EPR spectrum is largely on the low field side of $g = 2.00$. Three components can be easily identified as undergoing changes leading to a change in the EPR spectrum following 200 K illumination and subsequent dark storage at 77 K. $Y_D^{\bullet+}$ has a narrow ($\sim$2.5 mT wide) EPR spectrum, it cannot be the signal showing modulation 50 mT from $g = 2$.

$Q_A^{\bullet-}$ has a broader spectrum but is centred at $g = 1.9$ with little signal intensity on the low field side of $g = 2$. The Q_A site is predicted to be very hydrophobic [14], it therefore seems unlikely that water would be in direct contact with the site to give rise to the modulation seen in these experiments. We have previously shown using ENDOR that protons hydrogen bonded to $Q_A^{\bullet-}$ can be exchanged in ^2H_2O in samples treated with cyanide. H-bond protons would not be expected to give rise to interactions observed as Larmor frequency modulation. $Q_A^{\bullet-}$ has been reported to have a pH dependent redox potential in titrations of some types of preparation, but not in others (reviewed in [3]). It has been shown to function kinetically at its p*K*. Although the exchange experiment and some titration results show that the Q_A environment can exchange protons with the medium, the kinetic measurements suggest it is a slow, probably indirect, process which would not involve direct access of water to the site. The equal distribution about $g = 2.00$ and the line width over which the modulation is observed also suggest $Q_A^{\bullet-}$ is not the modulated signal.

The multiline Mn signal has a line width in excess of 150 mT centred at $g = 2.00$, with most of the signal intensity within 50 mT above and below $g = 2.00$, it is therefore the most likely candidate as the signal showing modulation. The weak modulation seen in the dark adapted samples has similar field distribution. The very slow decay of the modulation on storage in the dark on ice is similar to the behaviour of S_0. It may be that this modulation in the dark samples reflects interaction of water with S_0. It is unlikely that the modulation seen in the dark adapted sample reflects interaction with the component seen in the S_2 state as S_2 would decay rapidly under these conditions.

Although the line width and distribution around $g = 2.00$ suggest the Mn complex gives rise to the modulated signal, the decay of the signal is not compatible with the behaviour of all of the S_2 multiline signal. A part of the multiline signal does not decay during the time period when the modulation is completely lost. The proportion of stable multiline signal is less than in the presence of methanol, but a large part of the signal remains after 4 weeks and is then stable for at least a further 8 weeks. The light induced modulation is completely decayed after 4 weeks.

The results, in agreement with our previous experiments [12], suggest that the samples contain Mn centres in two different environments which give rise to a multiline EPR spectrum. In one environment the centres decay to an EPR silent state during storage at 77 K. Centres in the second environment are stable during 77 K storage. The fraction of stable centres seen in these experiments is less than in experiments in the presence of methanol [12]. However, in these experiments a fraction of centres are in the state giving rise to the $g = 4.1$ signal of the Mn complex (data not shown). Addition of methanol converts the $g = 4.1$ component to a multiline component. It seems likely that the centres which give rise to the $g = 4.1$ signal are converted by methanol to centres which give rise to the stable inaccessible multiline signal, increasing the apparent proportion of stable centres.

The existence of two different multiline signals, indicating the existence of two different Mn environments, has been suggested by Smith and Pace [15], observing the effects of methanol on the signal, and by Boussac [16] observing the response of the signal to infrared illumination. XAS also indicates the presence of two Mn environments, with two Mn μ-oxo-bridged structures with different orientations in the membrane [17] and responses to Ca depletion [18].

The experiments do not allow an unequivocal description of

the circumstances giving rise to these two environments. The parallel behaviour in terms of stability of the modulated signal seen in ESEEM, and the less stable multiline component in the EPR experiment, would support the proposal that the Mn in this environment is accessible to water, while the stable multiline component is not accessible. This might arise in two ways. The samples may contain photosystem II centres with a single Mn centre in two different conformations, or the Mn complex in each photosystem II centre has two distinct components.

The work of Wydrzynski's group [6] shows that water is bound to the water oxidation complex, and can exchange with the aqueous environment in S_3. The exchange implies that the water oxidation site has two states with, and without, water bound. The experiments described in this paper show that water has access to the Mn complex in S_1, as interaction with water is detected in samples converted to S_2 at 200 K. If there is a single Mn centre with four Mn atoms in a single complex the existence of two conformations could explain the present results. Freezing samples in S_1 would result in a mixture of photosystem II centres in each state. In the present experiments the fraction without water would be seen as having a stable multiline signal, and no modulation due to water. The fraction of centres with water bound would show modulation and have an unstable multiline signal on storage. This type of model would require a large fraction of centres without water bound. A large fraction of the multiline signal is stable, particularly in the presence of methanol, but is not in contact with the aqueous environment. It would also require that methanol affects the EPR spectrum of the water free centres, but is in direct contact only with water bound centres, or centres from which it displaces the water.

The second model requires that the Mn complex has two components in different environments in the membrane. In the S_2 state the oxidising equivalent may be located on either Mn component to give rise to a multiline EPR signal. The distribution on freezing representing the redox equilibrium between the two centres. This equilibrium may be altered by methanol. One 'inner' component would be in an anhydrous environment within the membrane. It would give rise to the stable multiline signal. The effect of methanol on the EPR spectrum would reflect gross effects on thylakoid structure rather than direct binding to the Mn. The second 'outer' component would be in an environment exposed to, but with limited access to, the aqueous environment. This outer Mn centre would also have water bound and water free conformations, but there would be no requirement for a large water free fraction. Methanol would have access to this component, but not to the 'inner' component. This explanation is compatible with the XAS results showing that there are two Mn dimers with different orientations to the membrane [18], and that only part of the Mn complex is affected by Ca depletion [19].

The experiments do not exclude the possibility that the ^2H modulated ESEEM signal is not one of the signals identified by CW EPR. A broad featureless signal from a component with a slower relaxation time than the Mn centre might be difficult to detect in CW EPR in the presence of the intense multiline signal. However, such a signal might be favoured by the pulsed EPR experiment where components with slow relaxation times are more readily detected than those with very fast relaxation. A signal with appropriate properties might arise from an organic radical coupled to a metal centre. Such a signal might reflect an oxidised ligand coupled to the metal centre as suggested by Pace and coworkers [19], or interaction between the Mn and Y_Z analogous to the state observed in the inhibited S_3' state [20,21].

The experiments do not provide direct evidence for water binding to Mn, but do show that water protons are within 3–5 Å of part of the Mn complex. The result is compatible with the proposal that the environment of the Mn complex provides the catalytic site for water oxidation as well as the accumulator for oxidising equivalents.

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