Review

Comparative studies of the S_0 and S_2 multiline electron paramagnetic resonance signals from the manganese cluster in Photosystem II

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Abstract

Electron paramagnetic resonance (EPR) spectroscopy is one of the major techniques used to analyse the structure and function of the water oxidising complex (WOC) in Photosystem II. The discovery of an EPR signal from the S_0 state has opened the way for new experiments, aiming to characterise the S_0 state and elucidate the differences between the different S states. We present a review of the biochemical and biophysical characterisation of the S_0 state multiline signal that has evolved since its discovery, and compare these results to previous and recent data from the S_2 multiline signal. We also present some new data from the S_2 state reached on the second turnover of the enzyme. ß 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Photosystem II (PSII) is a large membrane spanning enzyme in the thylakoid membrane in plants, algae and cyanobacteria [1,2]. It catalyses the light-driven reduction of plastoquinone using electrons from water, thereby oxidising two molecules of water to molecular oxygen. The active site in water oxidation, the water oxidising complex (WOC), is found on the luminal side of PSII and is mainly bound by the D1 reaction centre protein [1,3–5]. The WOC consists of four manganese atoms, the cofactors Ca^{2+} and Cl^−, and a closely interacting, redox-active tyrosine side chain, Y_Z (D1-Y161) [3,6–9].

During catalysis, the WOC cycles through five redox intermediates, the S states, denoted S_0 to S_4 [10] (Fig. 1). The S_0 to S_4 states are easily resolved kinetically while the transient S_4 state is rapidly reduced to S_0. The S cycle couples the one electron oxidation of the primary electron donor P680 to the four step oxidation of two water molecules to one dioxygen molecule.
After the absorption of a photon, the excited P680 reduces the acceptor complex. P680‡ is then rapidly reduced from Y Z in a pure electron transfer step. From studies of Mn-depleted systems [11–13] it is inferred that the oxidised Y Z loses a proton to a nearby base (thought to be His190 on the D1 protein) to form a neutral radical, Y c Z. The oxidised Y Z is re-reduced by the Mn cluster. It is debated [14,15] whether the reduction of Y c Z involves electron transfer from the Mn cluster and re-protonation of Y c Z from the same base, or if Y c Z is restored in a concerted electron and proton transfer reaction involving hydrogen atom transfer from Mn coordinated substrate water [16,17]. Several chapters in this volume deal with different aspects of this problem.

There are many other unresolved problems concerning the structure and function of the WOC. The structure of the Mn cluster is not known but recent X-ray crystallisation work holds great promise to soon resolve the most pressing matters (Orth, Witt, Zouni and Saenger, personal communication). The most advanced structural models are derived from X-ray spectroscopy, and a set of structures that fit the available data have been put forward [6,18–20]. The valence changes in the S cycle transitions are not unambiguously assigned, nor is the absolute Mn valence in the cluster known with certainty. It is generally agreed that the S 0 → S 1 and the S 1 → S 2 transition involve oxidation of the Mn cluster directly, whereas the S 2 → S 3 transition has been ascribed to Mn oxidation, ligand oxidation or partial substrate oxidation [6,19–23]. The chemistry leading to the formation of the oxygen-oxygen bond in the S 3 → S 0 transition is little understood although there are many detailed proposals in the literature (see for example [6,16] and chapters in this volume).

Electron paramagnetic resonance (EPR) spectroscopy is a useful method to gain structural insight, and to monitor the electron transfer processes in

Fig. 1. EPR spectra from the different S states of the WOC in PSII. Each quadrant of the figure shows spectra recorded from the S 0 to S 1 states as indicated by the Kok cycle in the centre of the figure. The broad S 1 spectrum (from spinach, supplied by Dr. A. Kawamori) and the structured S 1 spectrum (from a PSII core preparation from spinach depleted of its 23 and 17 kDa subunits [37], supplied by Drs. R.D. Britt and K. Campbell) are acquired in the parallel polarisation mode. The S 1 state spectrum is recorded in a Ca 2+ -depleted sample and shows the spin-coupled S 2 Y c Z signal. A parallel polarisation signal from the S 1 state (not shown) has also been observed (see text; [38,39]). The bars represent a field span of 500 G.
the WOC. Two of the S states have paramagnetic ground state EPR signals, and EPR signals have now been reported from all S states (Fig. 1). The best characterised EPR signals originate from S2, which gives rise to the hyperfine structured multiline EPR signal around $g = 2$ and a broad unstructured signal centred around $g = 4.1$ [24–26]. The S2 EPR signals have been extensively studied over two decades, and have been used successfully to probe biochemical phenomena such as inhibition patterns, cofactor dependence etc. There have been many studies dedicated to simulation of EPR signals from the Mn cluster, particularly from the S2 state, with a view to gain structural information on the WOC [27–33].

There is currently no consensus as to the number of manganese ions responsible for the S2 multiline signal, to the number and types of ligands around the manganese, to the valence of the manganese or to the spin state giving rise to the $g = 4.1$ signal. However, it is clear that this theoretical work is much needed as it continuously increases the knowledge about the Mn cluster in the S2 state. Even if the molecular structure for the Mn cluster will eventually (probably quite soon) become available in dark-adapted PSII, this will not solve critical issues concerning Mn interactions and ligand environment in the less accessible S states (S0, S2 and S3). Here, EPR spectroscopy is one invaluable tool. The recent discoveries of more EPR signals, also from other S states, will help in advancing the structural models based on simulations of a larger set of spectra.

The S1 and S3 states give rise to even spin EPR signals, which are best observed by parallel mode detection [34–39] (Fig. 1). In the formal S3 state, PSII also gives rise to a distinct EPR signal which is 100–240 G wide (Fig. 1) when it is inhibited by the removal of Ca$^{2+}$ or the addition of F$^-$/acetate ([3,8] and references therein). The spectrum originates from $Y^*_Z$ in magnetic interaction with the Mn cluster in the S2 state, and is therefore best known as the $S_2 Y^*_Z$ EPR signal [40–42].

The S0 state was long predicted to be paramagnetic and we first attempted more than 10 years ago to find EPR signals from S0 in oxygen evolving PSII membrane fragments (S. Styring and A.W. Rutherford, unpublished). At that time no EPR signals could be detected. This changed recently [43–45] with the discovery of a Mn hyperfine EPR signal from S0 (Fig. 1). The discovery is important since it provides a new spectroscopic probe to the Mn chemistry in fully functional PSII centres.

Here we review the first observation of the signal and our present progress in studies of the S0 EPR signal and its comparison with the S2 multiline signal. All stable S states can now be probed by EPR spectroscopy. This allows new experiments to characterise the individual S transitions and S states, for example pH changes, addition of isotope labelled substrates etc. Some attempts in this direction will also be described.

2. EPR signals from the Mn cluster that oscillate with S state

To study the entire S cycle with EPR spectroscopy, it is necessary to apply flash illumination from a powerful pulsed laser. Flash advancement normally results in a mix of S states after only a few flashes. In part, this dephasing can be avoided by application of a preflash protocol [43,46] that synchronises almost 100% of the PSII centres in the S1 state. It is crucial that the preflash and the following dark adaptation are carried out in the absence of any added exogenous electron acceptor. This shortens the time for complete deactivation of the S2 state thereby avoiding any reduction of $Y^*_D$ during the dark period. The electron acceptor, which is needed to accomplish optimal turnover conditions, is then added after dark adaptation for 15 min. The appropriate number of flashes, additions of reagents in different S states etc., is then immediately applied prior to freezing the EPR samples.

When this flash protocol is applied to intact PSII, the multiline EPR signal (as well as the $g = 4.1$ signal) from S2 oscillates well with flash number. It is big on the first flash and returns on the fifth flash. The oscillation has been repeated in PSII from many organisms and in many different buffer and cryoprotectant systems (reviewed in [3]).

Our intention was to study the EPR properties of the S0 state under different biochemical conditions. Methanol is often used to dissolve electron acceptors and is known to be harmless (when ≤ 5%) for PSII electron transport measured as oxygen evolution, variable fluorescence etc. However, alcohols have
long been known to influence the EPR signals from the S₂ state in a complicated (often unpredictable) manner and therefore electron acceptors used in EPR studies have often been dissolved in non-alcoholic solvents like DMSO. Methanol, for example, has large influence on the EPR signals from the S₂ state ([47], and see also below). We therefore decided to test the effect of methanol on the S₀ state and applied our flash protocol to PSII enriched samples in the presence of methanol. Fig. 2A shows a typical example of the EPR signals obtained from a flash series obtained with this protocol.

The first flash induces the S₂ multiline signal. This decreases with the second flash and has totally disappeared after three flashes. However, the disappearance of the S₂ state multiline is not immediately obvious since the three flash sample also contains a multiline signal (Fig. 2A). Careful examination reveals that this signal is very different from the S₂ multiline signal (compare the peak positions on the two signals, see below). The new signal is largest on the third flash when approx. 65% of the PSII population is in the S₀ state. It is also observable after four flashes (35% S₀). Thus, both multiline EPR signals oscillate with a period of four, and can therefore be assigned to the WOC. The new signal is formed after three flashes, and can consequently be assigned to the S₀ state (Fig. 2B). This is corroborated by its greater stability, compared to the S₂ multiline [43], which is typical for the S₀ state.

The S₀ EPR signal was discovered independently by us [43] and Messinger et al. [44,45]. Messinger and coworkers first observed the signal in a system where the WOC had been reduced by the addition of hydroxylamine [44]. In this case, a redox state denoted S₋₁ was formed. This was converted to the S₀ redox level by illumination in the presence of DCMU. The resultant Mn hyperfine signal, observed in the presence of methanol, was assigned to the S₀ state (in this case designated S₀* to mark that the state is achieved by chemical reduction prior to illumination). It closely resembles the signal observed by us (Fig. 2C) [43]. Messinger et al. were subsequently also able to generate the S₀ signal during flash advancement [45]. In this case, they used a lower PSII concentration than we and needed to concentrate their sample by centrifugation after the flash treatment. This increases the time between formation of the S₀ state and the actual freezing of the EPR sample from 1 s [43] to more than 30 min [45]. Potentially, this is of great importance for the characterisation of the S₀ state since the magnetic properties change during the first minutes after the formation of S₀ ([21]; Peterson and Styring, in preparation).

Fig. 2. (A) EPR spectra recorded in PSII enriched membranes poised in the different S states by laser flashes as described in the text. The samples contain 4 mg Chl/ml and 3% methanol (v/v). (B) The signal amplitude of the S₂ multiline signal (open triangles) and the S₀ EPR signal (filled triangles) as a function of flash number. The oscillations are simulated (dashed lines) assuming 10% S₀ and 90% S₁ in the preflashed samples and 13% misses on each flash. (C) Comparison at high resolution of the S₀ signal (solid line) and the S₂ multiline signal (dashed line). The spectra are illuminated-dark difference spectra. The S₀ spectrum was detected at high power (57 mW), where the S₂ multiline is almost completely saturated. A weighted amount of the S₂ multiline signal (10%) has been subtracted from the S₀ spectrum presented. The S₂ multiline signal has been scaled down four times compared to the S₀ signal. Reproducible edge peaks from the S₀ signal have been amplified two times and a splined baseline has been subtracted (insets). The Y₁⁺ radical at g = 2 has been cut out of both spectra.
3. Characteristics of the \( S_0 \) signal

Fig. 2C shows the spectra of the \( S_0 \) and \( S_2 \) multiline EPR signals in detail. The \( S_0 \) signal is approx. 20% wider than the \( S_2 \) multiline signal (2200 G compared to 1850 G). It has weaker amplitude in the central parts of the spectrum, but has pronounced intensity further out, including small, reproducible peaks far out on the edges. These peaks are enlarged and inset in Fig. 2C. In the \( S_0 \) spectrum presented in Fig. 2C we identify about 20 peaks on either side of the \( g = 2 \) region of the spectrum. Also, the separation of the major peaks varies across the \( S_0 \) spectrum. On average, the separation is smaller than that of the \( S_2 \) multiline, 82 G compared with 89 G in the \( S_2 \) state signal [48].

In \( S_2 \), the Mn cluster gives rise to both the multiline and the \( g = 4.1 \) signal (Fig. 1). The relative proportion between the multiline and the \( g = 4.1 \) signal is dependent on sample preparation, and an inter-conversion between the two signals can be induced by infrared illumination [49]. We have searched over a broad temperature interval for an \( S_0 \) signal in the high \( g \) region to find a signal corresponding to the \( S_2 \ g = 4.1 \) signal. At present we have not found any EPR signal from \( S_0 \) except for the multiline signal around \( g = 2 \) described in Fig. 2.

Although it is very difficult to accurately simulate the \( S_0 \) EPR signal, the relatively large spectral width allows conclusions about the oxidation state of the manganese ions in the WOC to be drawn. From X-ray spectroscopy and from spectral simulations of EPR spectra, the Mn oxidation states in the \( S_2 \) state are likely to be \( \text{Mn}^{III}\text{Mn}^{IV} \) or \( \text{Mn}^{II}\text{Mn}^{IV} \). It is agreed that Mn oxidation occurs on the steps from \( S_0 \) to \( S_2 \) [3,18–23]. Thus, the likely oxidation state for at least one pair of Mn in \( S_0 \) is \( \text{Mn}^{III}\text{Mn}^{III} \). Such a system may be modelled by assuming an effective spin Hamiltonian where the intrinsic hyperfine interactions are scaled by projection operators, as described in [43] (see also [29,50]). Assuming the same magnitude of the intrinsic hyperfine interaction for each manganese ion, regardless of oxidation state, the width of the spectrum, based on the projection operators for the two systems, is predicted to be 22% larger for a \( \text{Mn}^{III}\text{Mn}^{III} \) dimer than for a \( \text{Mn}^{III}\text{Mn}^{IV} \) dimer [31,43]. This matches well our observed spectral widths of the \( S_0 \) and \( S_2 \) multiline signals, supporting the assignment that one of the manganese ions in the \( S_0 \) state is \( \text{Mn}^{II} \). The presence of \( \text{Mn}^{II} \) in \( S_0 \) is also strongly supported by recent X-ray spectroscopy measurements [51].

4. Methanol effects on the \( S_0 \) and \( S_2 \) EPR signals

Methanol induces a multiline \( g = 2 \) signal in \( S_0 \). In \( S_2 \), the multiline signal is observable in the absence of methanol, but is enhanced by methanol (see [43,45,47,52,53] and references cited therein). Upon the addition of methanol, the \( S_2 \) signal at \( g = 4.1 \) decreases as the \( S_2 \) multiline increases.

We have studied the concentration dependence for the methanol induced changes of the \( S_0 \) and \( S_2 \) multiline signals and the \( S_2 \ g = 4.1 \) signal [52,54]. The methanol concentration at which half of the signal intensity is observed ([MeOH]_{1/2}) was found to be close to 0.2% (approx. 50 mM) for all three signals (Fig. 3). This suggests a similar binding interaction of methanol with the Mn cluster in the \( S_0 \) and \( S_2 \) states ([52]; see also [53]) (Fig. 3).

In a synthetic, mixed valence \( \text{Mn}^{III}\text{Mn}^{IV} \) complex, methanol was shown to replace water and ligate directly to the \( \text{Mn}^{III} \) ion [55]. Whether this is the case in the WOC as well is an interesting issue. The concentration of methanol used in our experiments does

![Fig. 3. Methanol concentration dependence of the \( S_0 \) (circles) and the \( S_2 \) multiline (squares) and \( g = 4.1 \) (triangles) signals. The amplitudes of the signals are calculated as in [52] for the \( S_0 \) and \( S_2 \) multiline signals, while the \( g = 4.1 \) signal amplitude is measured as peak to trough height. The total change in the amplitude is normalised over the investigated concentration range [52].](image-url)
not interfere with water oxidation which indicates that methanol does not bind to the substrate water site. However, it is likely that methanol binds to Mn in PSII, and ESEEM spectroscopy in the presence of $^2$H-labelled methanol and ethanol [53] showed that the Mn-to-alcohol distance in the S$_2$ state was 2.9–4.1 Å (depending on the structural model used). If this is true for the S$_2$ state, it should most likely also apply for the S$_0$ state, since our studies indicate similar methanol binding in the two S states.

Experiments in the presence of other alcohols [56] have been unsuccessful in producing the S$_0$ state multiline signal, even though some of them have a similar effect to methanol on the S$_2$ state. Thus, only methanol seems to be able to induce the well resolved S$_0$ multiline in plant PSII. The situation is different in PSII from _Synechococcus elongatus_ [111], where Bous-sac et al. [57] have observed the S$_0$ signal without the addition of methanol or any other alcohol. In the absence of methanol, Messinger et al. observed a broad EPR signal lacking hyperfine structure in samples given three flashes. This signal was also attributed to the S$_0$ state [45] but we have not been able to confirm this hypothesis. A broad feature with variable amplitude is often present in different S states. In order to assign such a broad feature to the Mn cluster in the S$_0$ state (or any other S state), this feature needs to be shown to oscillate with flash number. We have so far failed to do so and, in our view, the existence of a broad feature in the S$_0$ state still remains to be demonstrated. Also, this issue is probably better resolved by field-swept pulsed EPR, which detects the true EPR absorption in the S$_0$ state. Preliminary results (not shown; Peterson, Högblom and Styring, in preparation) show that the field-swept pulsed S$_0$ EPR spectrum in the presence of methanol is similar in intensity to the spectrum from the S$_2$ multiline signal. In contrast, in the absence of methanol, there is very little microwave absorption in the S$_0$ state. Similar results were mentioned (but not shown) by Boussac et al. [57]. This suggests that there is little, if any, S$_0$ multiline signal present in the absence of methanol, since the unmodulated ESE spectrum would reveal also a very broad signal if it was really present.

It is likely that the methanol effect observed in the different S states is caused by direct binding of methanol to manganese [52,53], but secondary effects of methanol on the protein environment cannot be excluded [52]. It has been shown that methanol modifies the EPR spectra in several S states by increasing the energy gap between the ground state and the first excited state of the system (see [48] and references therein). If this also applies to the S$_0$ state, it may be that the exchange coupling in the absence of methanol is very weakly antiferromagnetic, or even ferromagnetic [48]. This would give rise to a system that is difficult to detect with X-band EPR. A plausible explanation for such a situation is that the ground state of S$_0$ is a high spin state in the absence of methanol. The effect of methanol would then be to stabilise the S = 1/2 state as the ground state. This hypothesis will not be confirmed until a high-$g$ EPR signal is detected from S$_0$ in the absence of methanol.

5. Temperature dependence of S$_0$ and S$_2$ EPR signal amplitudes

The temperature dependence of the relaxation properties of the S$_0$ EPR signal has been compared to that of the S$_2$ multiline signal in the presence of methanol [48,58]. The unsaturated amplitude ($I_0$) of the two EPR signals were determined between 4 and 25 K. In Fig. 4, $I_0$ of the S$_0$ and S$_2$ multiline signals are plotted versus the reciprocal temperature. The S$_0$ signal appears to follow Curie dependence in that it
is linear over the measured temperature range, and clearly extrapolates to zero. There appears to be no thermally accessible excited spin state under these conditions. This behaviour is different from the temperature dependence of the \( S_2 \) multiline signal, which is linear in the interval 4–15 K, but does not extrapolate linearly to zero. The \( S = 1/2 \) spin state that gives rise to the \( S_2 \) multiline signal has a closer-lying excited state signal, that is, it is thermally accessible. However, in the presence of alcohol (methanol for \( S_0 \)) neither state exhibits an excited state EPR signal. Methanol apparently has a greater effect on the exchange interaction and hence the temperature dependence of the \( S_2 \) multiline signal, which provides information about the immediate surrounding of the Mn cluster. The powers of half saturation, \( P_{1/2} \), of the \( S_0 \) and \( S_2 \) multiline signals show different variations with temperature [58]. Below 8 K, higher applied microwave power is required to saturate the \( S_0 \) signal compared with the \( S_2 \) multiline, and at higher temperatures, the \( S_2 \) multiline requires more power.

The variation of \( P_{1/2} \) with temperature is indicative of the nature of the relaxation processes involved. \( P_{1/2} \) is a complex observable, proportional to the spin-spin relaxation rate, \( T_2^{-1} \), and the spin-lattice relaxation rate, \( T_1^{-1} \). \( T_1 \) is dependent on the interactions between a paramagnetic centre and its surroundings and can therefore provide information about subtle interactions between the Mn cluster and its immediate environment which otherwise would be very difficult to obtain. The temperature dependence of \( T_1 \) can be used as a reporter on such interactions. \( T_1 \) is best studied directly by pulsed EPR, but if the temperature dependence of \( T_2 \) is small, \( P_{1/2} \) can also be used as a reporter. In the Mn cluster, \( T_2 \) is dominated by inhomogeneous broadening, and is therefore not expected to contribute to \( P_{1/2} \). The relaxation rate of the \( S_0 \) signal is very fast which makes it difficult to induce a spin echo above 5 K. Therefore we chose to study \( T_1 \) processes by monitoring \( P_{1/2} \) as a function of temperature. Previous studies of the \( S_2 \) multiline yielded similar results from pulsed \( T_1 \) measurements [62] and conventional \( P_{1/2} \) measurements [47,63–65], with only slightly stronger temperature dependence of the pulsed data, confirming that \( T_2 \) variation does not contribute significantly to \( P_{1/2} \) variation in the \( S_2 \) state. We assume that the same holds for the \( S_0 \) state, and have analysed our \( P_{1/2} \) data as if they contain predominantly \( T_1 \) information.

The spin-lattice relaxation rate, and its temperature dependence, is determined by the mechanism
by which the lattice accepts the excess energy. There are three $T_1$ relaxation processes: the direct process, the Orbach process, and the Raman process. The first alternative is ruled out in our temperature range, as it typically dominates below 2 K. Fig. 5 shows fits of our $P_{1/2}$ data to an Orbach process (Fig. 5A) or a Raman process (Fig. 5B).

The Orbach mechanism represents thermal relaxation via an excited spin state of the system. This gives rise to an exponential temperature dependence of the relaxation rate:

$$1/T_1 \propto e^{-\Delta/k_BT}$$

(1)

where $\Delta$ is the energy gap from the ground state manifold to the excited state, $T$ is the temperature, and $k_B$ is the Boltzmann constant. The fits of the data to Eq. 1 (Fig. 5A) require $\Delta$ values of $(30 \pm 2)$ cm$^{-1}$ for $S_2$ and $(17 \pm 1)$ cm$^{-1}$ for $S_0$. [58].

Through the Raman mechanism, the spin system is relieved of excess energy through the scattering of a phonon in the surrounding protein lattice. The probability of such a process taking place is low, but the Raman process gains strength by its ability to utilise a large part of the vibrational spectrum of the lattice. If the ground state is isolated, and there is no thermally accessible excited state, the Raman mechanism will dominate. The Raman relaxation rate varies with temperature as

$$1/T_1 \propto T^n$$

(2)

where $n$ typically takes on values between 3 and 9. The fit of our data to Eq. 2 (Fig. 5B) yields a $T^{4.1 \pm 0.3}$ temperature dependence of $P_{1/2}$ of the $S_0$ signal and a $T^{6.8 \pm 0.2}$ temperature dependence of $P_{1/2}$ of the $S_2$ signal [58].

The fits of our data to Eqs. 1 and 2 are equally good (Fig. 5), and do not discern between a Raman and an Orbach relaxation mechanism. However, we can analyse the $P_{1/2}$ data in relation to the temperature dependence of the initial slope, determined earlier (Fig. 4). If an Orbach analysis is valid, the size of the energy gaps obtained should be in agreement with the $J$ couplings determined by the Curie plots. The Curie plots of Fig. 4 yield a $J$ coupling in $S_0$ more than twice as strong as that in $S_2$. This predicts a gap to the excited state that is much larger in $S_0$ than in $S_2$. This is contradictory to the gaps obtained from an Orbach analysis of the relaxation of the two signals, which predicts a gap of $17$ cm$^{-1}$ in $S_0$ and $30$ cm$^{-1}$ in $S_2$. Thus, the Orbach process cannot be responsible for the relaxation of both the $S_0$ and the $S_2$ multiline signals. At least one of the signals relaxes via a Raman process.

The gap of $30$ cm$^{-1}$ in $S_2$ derived by an Orbach fit is in very good agreement with the results from other groups studying the $S_2$ multiline signal [47,62,63,65]. This asserts the reproducibility of these investigations; the variation of $P_{1/2}$ with temperature does not seem to vary with preparation procedures or minor additions. In contrast, the Curie plots obtained by different groups [47,60,61,63,64] differ markedly from each other, and from that obtained by us [48]. This indicates that the magnitude of the exchange interaction in the Mn cluster is sensitive to preparation procedures and sample additions, while the $P_{1/2}$ behaviour is not. Perhaps the Orbach process is not the correct description of the $S_2$ multiline relaxation.

If both the $S_0$ and $S_2$ multiline signals relax via a Raman mechanism, this may have some interesting implications for the protein holding the Mn cluster. Stapleton and coworkers have worked out a model for metal containing proteins, that successfully couples the protein structure to the Raman relaxation rate of the metal centre EPR signal ([66]; see also [58]). According to this model, the relaxation rate is
affected by changes of secondary structural elements and in the bridging between different groups in the surrounding protein. Applying this to our $P_{1/2}$ data, such differences seem to be present between the $S_0$ and $S_2$ states of the WOC. We have found a much weaker temperature dependence of the relaxation rate of the $S_0$ signal than of the $S_2$ signal, $T^{4.1}$ and $T^{6.8}$ respectively, suggesting a more compact conformation in $S_2$ than in $S_0$ [58]. The effect is consistent with a tightening of the surrounding protein with increasing oxidation state. Such changes are not unlikely in light of EXAFS results [6,23] that show significant, albeit small, changes in Mn-Mn distances between the $S_0$ and $S_2$ states.

Another possibility is that the $S_2$ multiline relaxes via an Orbach mechanism, as often has been assumed, while the $S_0$ signal relaxes via a Raman mechanism. If this is the case, the comparative model of Stapleton and coworkers cannot be applied. Rather, the $S_2$ signal relaxes via the first excited state of the system, which is thermally accessible according to Fig. 4A. In $S_0$, this state is inaccessible, and relaxation is forced to occur via a Raman process. An Orbach relaxation process in $S_2$ does not account for the varying Curie fits from different laboratories, since the Orbach analysis gives almost identical values of the energy gap to the first excites state. It is corroborated, however, by pulsed $T_1$ measurements [62], that yield a better fit to Eq. 1 than to Eq. 2.

7. Differences between the $S_2$ multiline after one and five flashes

We have prepared the $S_2$ state by a preflash treatment followed either by one flash or by five flashes before freezing. The signal amplitude after five flashes is approx. 50% of that after one flash, due to dephasing of the PSII centres. With methanol present, the multiline hyperfine patterns from the two $S_2$ samples are identical. Their saturation behaviour, however, differs. At 5 K, the multiline signal after one flash has a $P_{1/2}$ of 21 mW, whereas the multiline after five flashes has a $P_{1/2}$ of approx. 122 mW. Fig. 6 shows the microwave saturation curves at different temperatures of the $S_2$ multiline signal obtained after one and five flashes respectively. It is immediately clear that the multiline signal in the five-flash sample relaxes faster than the multiline after one flash throughout the temperature region studied.

This subtle difference between the $S_2$ state in the first and the second turnover is intriguing; it suggests that the Mn cluster changes during the first turnover. We observe no spectral differences between the one-flash and the five-flash multiline signals in the presence of methanol. However, preliminary observations indicate that there exist direct spectral differences with no alcohols present: the one-flash multiline signal has stronger central peaks than does the five-flash multiline signal (S. Peterson and S. Styring, in preparation). The differences we detect between the $S_2$ state in the first and second turnover are probably related to the reported inhomogeneity of the $S_2$ state multiline signal produced by illumination at 200 K. Evans et al. [67] have identified a narrower multiline component that shows ESEEM from $^2$H-methanol, and a broader component that does not. These are probably the same two components that were distinguished by Boussac [68], who showed that the broader multiline converts to the $g = 4.1$ signal upon infrared illumination more readily than the central peaks do. This inhomogeneity can also explain the different $P_{1/2}$ values within the $S_2$ multiline signal reported by Pace et al. [47]: the edge

![Fig. 6. Microwave power saturation of the $S_2$ multiline signal after one flash (filled circles) and after five flashes (open circles), at 4.2 K (bottom traces), 5 K (middle traces) and 7 K (top traces). The data have been normalised to the same initial slopes. The fits of the data represent $P_{1/2}$ values for the one-flash data of 3.2 mW at 4.2 K, 21 mW at 5 K, and 80 mW at 7 K. For the five-flash data, $P_{1/2}$ is 14 mW at 4.2 K, 122 mW at 5 K, and approx. 350 mW at 7 K.](image-url)
peaks were shown to be more readily saturated than the central peaks in the presence of methanol.

The inhomogeneity of the multiline signal has been proposed to arise from different PSII populations [68] or from different forms of the formal S2 oxidation state [67]. Our observation of potentially correlated changes occurring during turnover of PSII alter the picture. It is possible that one of the components oscillates better than the other one, thus dominating the spectrum after five flashes. However, we find this unlikely since the oscillation of the multiline signal is very good (Fig. 2). This would not be the case if a large fraction of PSII did not oscillate with flash number. Instead our data suggest that the PSII centres change during the first turnover. We are currently investigating this further in an attempt to identify the changes giving rise to the differences in the multiline signal. If the S2 state is different in the second turnover than the first, the ‘second cycle S2’ is possibly of greater functional relevance than the ‘first cycle S2’. The difference we detect may be indicative of a light induced alteration of the WOC. This was first observed by Brudvig and coworkers who suggested it to reflect a transition from a resting to an active form of WOC [69], similar to what is known to occur in cytochrome oxidase.

8. pH induced effects on the S0 and S2 multil ine EPR signals

Many of the catalytic events in PSII are pH-dependent and the optimal pH range for oxygen evolution is quite narrow. Much focus has been directed to the oxidation and reduction of both YZ and YD that are pH-dependent reactions [17,70].

It is also likely that many properties and reactions in the Mn cluster are pH-dependent, and we have developed a novel approach where we can study the effect of pH directly on the S0 and S2 states [71]. In our experiments, the pH is altered after the exciting flashes (three flashes: S0 and one flash: S2). The graphs show the change in the amplitude of the S0 and S2 state signals. The solid lines are the best fits of the data to two pH values. The two pH values, for the amplitude change, determined for each S state signal were $pK_1 = 4.2 \pm 0.2$ and $pK_2 = 8.0 \pm 0.1$ for the S0 multiline and $pK_1 = 4.5 \pm 0.1$ and $pK_2 = 7.6 \pm 0.1$ for the S2 multiline [71].

Fig. 7. The pH titration curves of the S0 (A) and S2 (B) state multiline signals. The pH was changed after the exciting flashes (three flashes: S0 and one flash: S2). The graphs show the change in the amplitude of the S0 and S2 state signals. The solid lines are the best fits of the data to two pH values. The two pH values, for the amplitude change, determined for each S state signal were $pK_1 = 4.2 \pm 0.2$ and $pK_2 = 8.0 \pm 0.1$ for the S0 multiline and $pK_1 = 4.5 \pm 0.1$ and $pK_2 = 7.6 \pm 0.1$ for the S2 multiline [71].

The signal amplitudes were almost recovered when the pH was changed back from alkaline pH to pH 6, while the recovery was less pronounced when returning from acidic pH. There are several different options for the decreased S0 and S2 multiline signals, but the reversibility shows that the S state itself was not lost during the titration. It is possible that the signal change involves titration of substrate water (or derivatives of water). If this was the case, the observation that the pH values are similar in S0 and S2 has important implications for the view of charge neutrality in the WOC. If there was a buildup of charge in the WOC during the turnover of the S states this would affect the pH values of any water bound to the Mn cluster in the S0 and S2 states [16,17]. Consequently, our data support the notion that charge neutrality is maintained during the oxidation of the WOC [71].
One possible explanation for the loss of signal amplitude at acidic and alkaline pH involves titration of amino acid residues interacting with the manganese ions. It is probable that ligands would titrate quite similarly in both S0 and S2. Attractive ligand candidates are His residues and carboxylate residues. Studies have shown that replacement of for example D1-His190 with other amino acid residues inhibits assembly of the Mn cluster. This indicates that D1-His190 is very important for the ligation of the Mn cluster [72–75]. D1-His190 is also thought to be hydrogen bonded to YZ [11–13,76,77]. The alkaline pKs values determined by us (8.0 and 7.6 for S0 and S2 respectively (Fig. 7; [71]) are similar to the pKs for His190 (pK 7–8) [11,12].

Another possibility is that the titration reflects interactions involving YZ [71]. A very close interaction between YZ and the Mn cluster has been observed in calcium-, or chloride-depleted, or acetate-treated samples subjected to illumination (see Section 1). After two flashes to such treated samples (i.e. the S1 state) a split signal originating from S2YZ can be trapped ([3,8] and references therein; [40–42]), showing that even though YZ may not ligate to the Mn cluster it can definitely affect the magnetic properties of the Mn cluster.

There is one further possibility: that the redox potential of the YZ/Y+Z couple changes with pH. The presence of a pH-dependent donor side equilibrium has been suggested previously [70,78,79]. It has also been shown that the YZ/Y+Z redox couple has a pH-dependent redox potential which decreases with 59 mV per pH unit as the pH is increased [80,81]. Consequently, if the YZ/Y+Z redox couple shows the same pH dependence, it is quite possible that the equilibrium S0YZ ⇌ S0+1YZ which is normally shifted strongly to the right could be shifted to the left at alkaline pH.

If the increased pH resulted in a decreased potential of YZ/Y+Z it is feasible that S2 might oxidise YZ, as they are separated by only 40–60 mV [70]. The most likely option would be that a free radical signal from YZ would become visible at the expense of the Mn-derived S2 state EPR signals. Any observable signal from YZ in the S1 state most likely would be observable as a free tyrosine radical in the EPR spectrum. We do not observe any additional amplitude in the radical region of the EPR spectrum during the pH titration [71]. Thus, if the manganese EPR signals were lost due to a lowered redox potential of YZ this does not result in a normal S1YZ state. Furthermore, in the S0 state it is highly unlikely that S0YZ is shifted to, in this case, S−1YZ, since YZ can be expected to be more oxidising than S0 also at pH 8.5 (at physiological pH, Y+Z has been estimated to be ≥ 250 mV more oxidising than the S0 state [71]). Consequently, we find it unlikely that redox changes directly involving YZ can explain our observed loss of the S0 and S2 multiline EPR signals at alkaline pH. This holds especially for the S0 multiline signal while it cannot be totally ruled out for the S2 multiline signal.

Further studies of the pH effects on the different S states may give more important information about the environment of the Mn cluster. Such studies are in progress in our laboratory with the approach we have developed (Geijer and Styring, in preparation).

9. Conclusions

For a long time EPR studies of the WOC were limited to the multiline and g = 4.1 EPR signals from the S2 state. The situation has changed and we can now study all S states with EPR spectroscopy. In this review we have focussed on the discovery of a multiline EPR signal from the S0 state. The temperature dependence of the S0 signal indicates that the signal originates from an isolated ground state. The relaxation behaviour of the multiline signals in S0 and S2 have been compared and the results suggest that the two signals relax via Raman mechanisms (although an Orbach mechanism cannot be excluded for S2). The relaxation mechanisms follow different temperature dependence and the results can be interpreted to originate from a less compact arrangement of the protein surrounding the Mn cluster in the S0 state than in the S2 state.

Subtle changes in the structure of the WOC are revealed by detailed comparison of the S2 multiline signal obtained after one flash (before the first turnover is completed) and after five flashes (during the second turnover of PSI1). The S2 multiline signals observed after one or five flashes respond differently to the addition of methanol to the sample, have slightly different hyperfine structure and relax quite
differently. This behaviour is coupled to a set of seemingly unrelated observations in the literature and is proposed to reflect a turnover-dependent conversion of the WOC to an activated form.

The discoveries of EPR signals from more S states than the S2 state have also allowed a new type of mechanistic EPR studies in which the binding of ligands to the WOC in different S states can be directly assessed. We describe two such studies in which the effect of the binding of two small ligands, methanol and \( \text{OH}^- \), are monitored. Methanol has an effect on the EPR signals from all S states. In both S2 and S0 the presence of methanol dramatically changes the EPR properties of the WOC. In S0, no EPR signal is observable in the absence of methanol. The concentration dependence for both effects is very similar which suggests that methanol binds to a site close to or at the Mn cluster which is not affected by the S state. The pH also has similar effects on the WOC in several S states. In the described experiments, pH is altered after the S state has been reached at optimal pH. In this case, the S2 and S0 multiline signals are both lost reversibly by increasing or decreasing the pH. The pK\(_a\) values for the loss of the two signals are remarkably similar.

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