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Methanol modification of the electron paramagnetic resonance signals from the S_0 and S_2 states of the water-oxidizing complex of Photosystem II

Zsuzsanna Deák [1], Sindra Peterson *, Paulina Geijer, Karin A. Åhrling [2], Stenbjörn Styring

Department of Biochemistry, Center for Chemistry, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

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Abstract

The Mn-derived electron paramagnetic resonance (EPR) multiline signal from the S_0 state of the water-oxidizing complex is observable only in the presence methanol. In the present study, we have characterized the effect of methanol on the EPR signals from the S_0 and S_2 states as well as on the EPR Signal II_{slow} originating from the $Tyrosine_D^{ox}$ radical. The amplitudes of the S_0 and S_2 multiline signals increase with the methanol concentration in a similar way, whereas the S_2 $g=4.1$ excited state signal amplitude shows a concomitant decrease. The methanol concentration at which half of the spectral change has occurred is $\approx 0.2\%$ and the effect is saturating around 5%. Methanol has an effect on the microwave power saturation of the S_2 multiline signal, as well. The microwave power at half saturation ($P_{1/2}$) is 85 mW in the presence of methanol, whereas the signal relaxes much slower ($P_{1/2} \approx 27$ mW) without. The relaxation of Signal II_{slow} in the presence of methanol has also been investigated. The $P_{1/2}$ value of Signal II_{slow} oscillates with the S cycle in a similar way as without methanol, but the $P_{1/2}$ values are consistently lower in the methanol-containing samples. From the results, we conclude that methanol modifies the magnetic properties of the S_0 and S_2 states in a similar way. The possible site and nature of methanol binding is discussed.

Keywords: Methanol; Manganese cluster; Photosystem II; Electron paramagnetic resonance

1. Introduction

Photosystem II (PSII) is a multisubunit enzyme complex located in the thylakoid membranes of algae and higher plants. Upon illumination, it oxidises water on the lumenal side, and reduces plastoquinone on the stromal side of the membrane (for recent reviews see [1–3]). PSII consists of more than 25 subunits [4], but only two of these, the D1 and D2 proteins, are directly involved in the photosynthetic electron transport reactions. The D1/D2 heterodimer binds the photosensitive electron donor P680, the

Abbreviations: Chl, chlorophyll; cw, continuous wave; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; MES, 2-[*N*-morpholino]ethanesulfonic acid; PPBQ, phenyl-*p*-benzoquinone; PSII, Photosystem II; SII_{slow}, Signal II_{slow}; WOC, water-oxidizing complex

* Corresponding author.

[1] On leave from the Institute of Plant Biology, Biological Research Center, H-6701 Szeged, P.O. Box 521, Hungary.

[2] Present address: Department of Chemistry, Faculty of Science, Australian National University, Canberra 0200, Australia.

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primary electron acceptor pheophytin, and the Q_A and Q_B secondary quinone electron acceptors. On the donor side of PSII, two redox active tyrosyl residues are found, called Y_Z (D1-Tyr161) and Y_D (D2-Tyr161). Although their positions are symmetrical in respect to P680, only Y_Z plays a role in the linear electron transport from substrate water to $P680^+$, while Y_D is an accessory electron donor.

Photosynthetic oxidation of water to molecular oxygen takes place in the water-oxidizing complex (WOC) on the donor side of PSII (for reviews see [1,5]). The WOC contains a cluster of four Mn atoms bound to the D1/D2 heterodimer. During illumination, the WOC cycles through five different redox states, entitled S_0–S_4. The subscript indicates the number of oxidizing equivalents stored in each state.

Photoexcitation of the primary electron donor P680 leads to the primary charge separation, after which $P680^+$ is rereduced by Y_Z, which in turn is reduced by the WOC, as the $S_i \rightarrow S_{i+1}$ transition occurs. Molecular oxygen is released, when four oxidizing equivalents have been accumulated, in the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. Only the S_1 state is stable in the dark; the S_2 and S_3 states decay to S_1 on a seconds time scale, and S_0 is oxidized to S_1 in minutes at room temperature.

The X-ray crystallographic structure of the WOC (or PSII) has not yet been determined, but models have been developed based on the large amount of spectroscopic and biochemical information available. An often discussed structural model [6] places the four Mn atoms of the WOC as a dimer of di-μ-oxo-bridged dimers (for review see [7]).

Electron paramagnetic resonance (EPR) is sensitive to unpaired electrons, and has proven useful in the investigation of the different S states (for reviews see [8–10]). The S_2 state gives rise to the broad, hyperfine-structured multiline EPR signal centered around $g = 2$ [11]. This signal resembles that of a mixed-valence dimer, and is thought to arise from a Mn(III)–Mn(IV) oxidation state of two of the Mn ions, while the other two Mn ions have the same valences (either Mn(III) or Mn(IV)). Whether only two or all four of the Mn ions are needed to sufficiently explain the physical properties of the EPR signal is disputed [12–15]. Another broad, but unstructured EPR signal from the S_2 state appears at $g = 4.1$ [16,17]. Two forms of this signal have been distinguished: a ground state signal induced by 130 K illumination in the presence of 30% ethylene glycol, and a signal induced by 200 K illumination with no alcohols present which has been assigned to an excited state [18] (this signal will therefore be denoted the 'excited state' $g = 4.1$ signal in this paper).

The S_0 state, being two electrons more reduced than the S_2 state, has long been predicted to be paramagnetic (for example [19]), and recently, an EPR signal from this state was discovered [20–22]. The S_0 state EPR signal is similar to the S_2 multiline signal (Fig. 1), but it is more than 2500 Gauss wide, compared to the 1850 Gauss of the S_2 multiline [23]. It also has different peak separation and relative peak intensities, with an average peak spacing of 82 Gauss, compared to 89 Gauss in the S_2 multiline [21], and relatively large intensities at the edges of the spectrum. These features are consistent with an antiferromagnetically coupled Mn(II)–Mn(III) oxidation state [20–22]. Temperature dependence studies of the S_0 state EPR signal indicates that it arises from an $S = {}^1/_2$ ground state with no thermally accessible excited state [23].

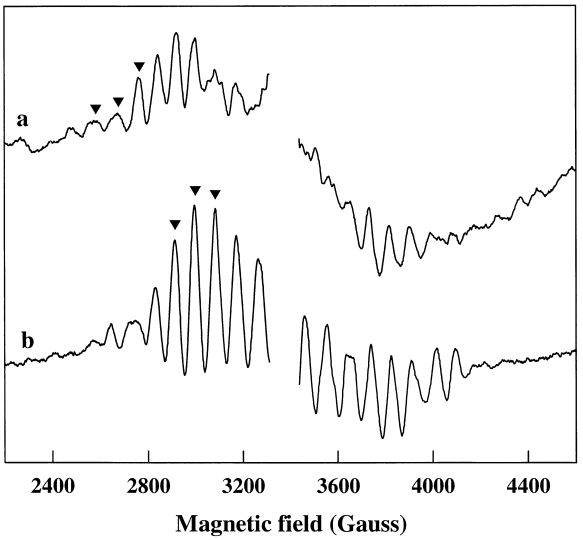

Fig. 1. The S_0 state (a) and S_2 state multiline (b) EPR signals recorded in samples given three and one flashes, respectively. The figure shows illuminated minus dark spectra. The S_0 spectrum may contain a small amount (5–10%) of S_2 multiline. The peaks used for evaluation of signal amplitudes are indicated with triangles. The region of the Y_D^{ox} radical has been omitted for clarity. EPR conditions: temperature 7 K, microwave power 14 mW (S_2 signal) and 56 mW (S_0 signal), microwave frequency 9.47 GHz, modulation amplitude 20 G, modulation frequency 100 kHz.

When oxidized, Y_D gives rise to a radical EPR signal, called Signal II_{slow} (SII_{slow}) [2]. Being located in the vicinity of the Mn cluster [24,25], Y_D^{ox} is sensitive to magnetic changes in the Mn cluster. The relaxation of SII_{slow} is enhanced in a way that reflects the relaxation of the WOC [19]. In the absence of Mn-originating EPR signals from most S states, SII_{slow} has been used to extract information about the WOC in these states [19,26].

An interesting and important characteristic of the S_0 signal is that it appears only in the presence of a few percent of methanol [21–23]. Alcohols have effect on the other S state signals, as well. The S_2 excited state $g = 4.1$ signal is very small or absent in the presence of 50% glycerol, 30% ethylene glycol, 5% ethanol [27], or 3% methanol [28], while these additions enhance the S_2 ground state multiline signal. The parallel polarized unresolved EPR signal with a g value of about 4.9 from the S_1 state [29] is not observable in the presence of 30% ethylene glycol or 3% methanol, although it was observed in a sucrose buffer supplemented by 50% glycerol [30]. Very recently, alcohol binding to the Mn cluster in the S_2 state of the WOC was investigated [31]. Using ESEEM spectroscopy and ^2H-labeled alcohols, direct evidence was provided that small alcohols (methanol and ethanol) ligate to the Mn cluster in the S_2 state.

In the present study, we have further characterized the effect of methanol on the PSII donor side EPR signals. The manganese EPR signals show amplitude variations as a function of methanol concentration, and the microwave power saturation of the S_2 multiline signal and SII_{slow} are also affected by the addition of methanol.

2. Materials and methods

PSII-enriched membranes were prepared from liquid-culture grown spinach as described in Pace et al. [28] and stored at −80°C in 400 mM sucrose, 20 mM MES pH 6.0, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, at about 10 mg Chl/ml. The membrane particles were diluted to 4 mg Chl/ml with the storage buffer and methanol, and transferred to calibrated EPR tubes. EPR samples with PSII in the S_0 and S_2 states were prepared according to Åhrling et al. [21]. One preflash was given to each sample followed by a 15–20 min dark incubation at room temperature to synchronise all the PSII centers in the S_1 state [32]. When used, the artificial electron acceptor phenyl-*p*-benzoquinone (PPBQ, 50 mM stock solution) dissolved in dimethyl sulfoxide (DMSO), was added at the end of the dark incubation to a final concentration of 0.5 mM. In control experiments, DMSO was added to the same final concentration (1% v/v). One minute after the addition, one or three exciting flashes were given, after which the samples were immediately frozen. This resulted in samples dominated by PSII centers in the S_2 or S_0 states, respectively. Due to an excitation miss factor of 15% per flash, the 1-flash sample contains 85% centers in the S_2 state and 15% centers in the S_1 state, and the 3-flash sample contains 61% S_0 centers, 33% S_3 centers and 6% S_2 centers. (The distribution is derived from the oscillation of the S_2 multiline amplitude in each series of flashed samples [19,21].) The S_1 and S_3 contributions do not affect our manganese EPR measurements, since these states do not give rise to conventional EPR signals. However, when measuring the microwave saturation of SII_{slow} in the flashed samples, one has to take into account that SII_{slow} is observable in all S states, with a different relaxation behavior in each. Thus, the measured SII_{slow} saturation curves represent sample-specific mixes, that were deconvoluted using the S state distribution of each sample.

The presence of 5% of methanol does not affect the oxygen evolving capacity (300–400 μM O_2/mg Chl/h) or the flash oxygen yield.

The saturating flashes were delivered by a Nd:YAG laser (6 ns, 350–400 mJ, 532 nm) at a computer-controlled flash frequency of 2.5 Hz. Low temperature continuous wave (cw) EPR spectra at X-band were recorded with a Bruker 380E spectrometer equipped with an Oxford Instruments cryostat. Data handling was done with the Bruker WinEPR and SigmaPlot 4.0 software.

3. Results

3.1. Methanol concentration dependence of the S_0 and S_2 state EPR signals

The hyperfine structure of the S_0 EPR signal ap-

pears only in the presence of a few percent of methanol [21–23]. A characteristic S_0 state EPR spectrum is shown in Fig. 1, along with the S_2 state multiline signal, displaying the differences as well as the similarities of the two signals. For comparison, both samples contain 5% (v/v) methanol. To quantify the S_0 signal, it is important to choose peaks that do not overlap with S_2 multiline peaks, to assure that the PSII centers in the S_2 state in the 3-flash sample ($\approx$6%, see Section 2) do not affect the results. The peaks we have used for amplitude analysis are indicated with triangles in Fig. 1.

The low-field regions of the EPR signals from the S_0 and S_2 states in samples with different methanol-concentrations are shown in Fig. 2. Very high microwave power (140 mW) was used to detect the S_0 signal, in order to enhance the S_0 signal and saturate away any contributions from the S_2 multiline, which is easier to saturate than the S_0 signal below 8 K [33]. Fig. 2A demonstrates that the hyperfine structure of the S_0 EPR signal is dependent on methanol concentration. Without methanol, hardly any hyperfine structure is observable (the detected structure is from contaminating S_2 multiline signal), but it is clearly visible above 0.1%, and has reached its maximum amplitude at 5% of methanol. Fig. 2B shows that the amplitudes of the peaks in the S_2 multiline signal, although not dependent on methanol for their appearance, also increase with methanol concentration. The S_2 multiline signal amplitude increases about 2.5-fold with increasing methanol concentration from 0 to 5%. As shown in Fig. 2C, the excited state $g=4.1$ signal from the S_2 state has its maximal amplitude in the absence of methanol and completely disappears in the sample containing 5% of methanol.

There is a broad underlying signal around $g=2$ in the S_0 state samples (Fig. 1, spectrum a) [21,22]. This signal shows up both in the presence and the absence of methanol [22] and its amplitude does not change significantly with methanol concentration (data not shown).

The methanol concentration dependence of the amplitude of the S_0 signal, the S_2 multiline and $g=4.1$ signals is shown in Fig. 3. The methanol-induced, total change of the signal amplitude is normalized to 100% for all three signals. Treating the interaction as an enzyme–substrate reaction, a hyperbolic fit was applied. From this, the methanol concentration at which half of the spectral change has occurred, $[MeOH]_{1/2}$, could be estimated.

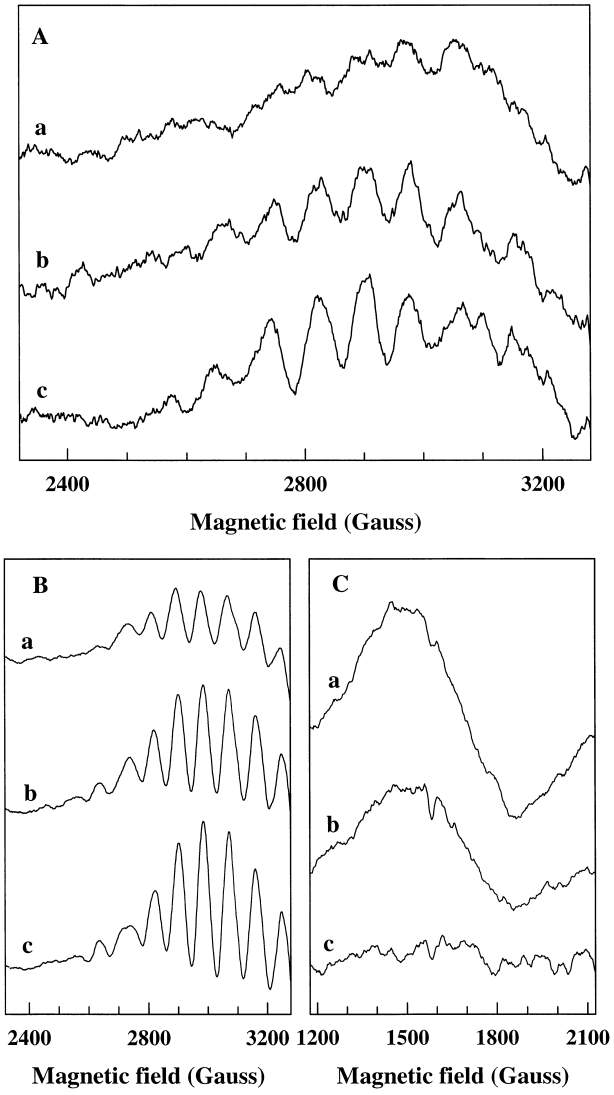

Fig. 2. EPR spectra from the S_0 (A) and S_2 (B,C) states in the presence of different concentrations of methanol. In A and B, the samples contain 0% (a), 0.5% (b) and 5% (c) methanol and are prepared in presence of 0.5 mM PPBQ dissolved in DMSO (1%). The spectra are linearly baseline corrected raw data, and only the low field region is shown. C shows the $g=4.1$ signal of the S_2 state from the samples with 0% (a), 0.2% (b) and 5% (c) methanol, prepared without PPBQ or DMSO. The spectra are baseline corrected illuminated minus dark spectra. EPR conditions: temperature, 5 (A), 7 (B), and 15 (C) K; microwave power, 140 (A), 14 (B), and 6 (C) mW; microwave frequency, 9.44 (A), 9.45 (B) and 9.47 (C) GHz; modulation amplitude, 20 G; modulation frequency, 100 kHz.

The methanol concentration dependence of the S_0 signal amplitude at two different measuring condi-

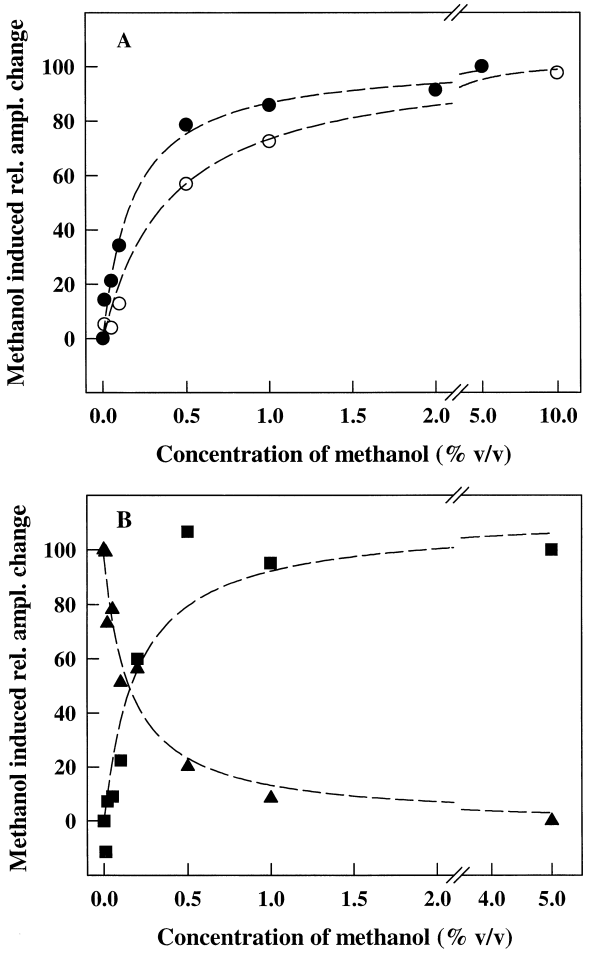

Fig. 3. Methanol concentration dependence of the S_0 (A) and the S_2 multiline (B, ■) and $g=4.1$ (B, ▲) signals. The amplitudes of the signals are calculated as the sum of the peaks indicated in Fig. 1 for the S_0 and S_2 multiline signals, while the $g=4.1$ signal amplitude is measured as peak to trough height. The total change in the amplitude is normalized over the investigated concentration range. The S_0 samples were prepared with (○) or without (●) PPBQ dissolved in DMSO (1%); the presented S_2 results are from samples without PPBQ and DMSO. EPR conditions: temperature, 5 (A, ○), 7 (A, ●), and 15 (B) K; microwave power, 140 (A, ○), 56 (A, ●), 14 (B, ■), and 6 (B, ▲) mW.

tions is shown in Fig. 3A. In samples prepared using PPBQ as electron acceptor, dissolved in DMSO (Fig. 3A, open circles; spectra in Fig. 2A) yields $[MeOH]_{1/2} \approx 0.40\%$. In contrast, when the S_0 signal was studied in the absence of PPBQ and DMSO (Fig. 3A, closed circles) we found $[MeOH]_{1/2} \approx 0.18\%$, which is significantly lower. This suggests that PPBQ and/or DMSO has influence on $[MeOH]_{1/2}$ which was studied in separate experiments (see below).

Fig. 3B shows how the amplitude of the $g=4.1$ signal decreases when the concentration of methanol is increased. Concomitantly the S_2 multiline signal increases. The $[MeOH]_{1/2}$ values are similar in these signals (multiline, $\approx 0.18\%$; $g=4.1$, $\approx 0.16\%$), indicating an interconversion between two states. These S_2 samples were prepared without PPBQ and DMSO, since the presence of PPBQ disturbs the observation of the $g=4.1$ signal, by inducing the EPR signals from Fe^{3+} Q_A [34,35] in the same area of the spectrum. We also measured the methanol influence on the S_2 multiline signal in the presence of PPBQ and DMSO. In this case we found $[MeOH]_{1/2} \approx 0.35\%$ (not shown in Fig. 3B) which is similar to what was found for the S_0 signal in the presence of these agents.

The $[MeOH]_{1/2}$ data of methanol concentration studies are summarized in Table 1. We have measured $[MeOH]_{1/2}$ for both the S_0 and the S_2 signals in the absence and presence of PPBQ dissolved in DMSO. In the absence of PPBQ, the $[MeOH]_{1/2}$ was $\approx 0.2\%$ for all signals. The $[MeOH]_{1/2}$ of the S_0 and S_2 multiline signals was increased to 0.4% by the addition of PPBQ dissolved in DMSO. A slight increase was also found for the $g=4.1$ signal, although this signal is difficult to quantify in the presence of PPBQ, as explained above. Thus, it seems that all signals are influenced in the same way by methanol. An additional unexpected effect was that $[MeOH]_{1/2}$ was doubled in the presence of PPBQ dissolved in DMSO. In order to discriminate between effects of PPBQ and DMSO, $[MeOH]_{1/2}$ of the S_2 multiline was determined with 1% DMSO but no PPBQ present in the sample. This yielded $[MeOH]_{1/2} \approx 0.43\ (\pm 0.05)\%$. We conclude that the observed increase in $[MeOH]_{1/2}$ is induced by the DMSO and not by PPBQ.

3.2. *Effect of methanol on the microwave power saturation of the S_0 and S_2 multiline signals and SII_{slow}*

Fig. 4 shows the microwave power saturation at 7 K of the S_0 and S_2 multiline signals in the presence of 3% methanol, and of the S_2 multiline with no methanol in the sample. The S_0 signal is not observ-

Table 1
Methanol concentration at which the half of the spectral change has occurred ($[MeOH]_{1/2}$)

EPR signal	$[MeOH]_{1/2}$ (% v/v)	
	Without PPBQ/DMSO	With PPBQ/DMSO[a]
S_0 multiline	0.18 ± 0.03[b] (7 K, 56 mW)[c]	0.40 ± 0.07 (5 K, 140 mW)
S_2 multiline	0.23 ± 0.10 (7 K, 14 mW)	0.35 ± 0.08 (7 K, 14 mW)
	0.18 ± 0.08 (15 K, 14 mW)	
S_2 state $g = 4.1$	0.16 ± 0.04 (15 K, 6 mW)	0.20 ± 0.08 (10 K, 140 mW)

[a]Final concentrations of PPBQ is 0.5 mM and of DMSO 1% (v/v).
[b]The $[MeOH]_{1/2}$ value is a regression coefficient of a hyperbolic fit, followed by standard error, which estimates the uncertainties in the value of the regression coefficient.
[c]The measuring conditions of the EPR signals: temperature and microwave power.

able without methanol, consequently the same comparison cannot be made. As the cw microwave power is increased, the signal amplitudes first increase linearly, then reach their maxima as the signals are saturated. This is described by

$$I = I_0 P^{0.5}/(1 + P/P_{1/2})^{0.5b} \quad (1)$$

where I is the measured signal amplitude, P the applied microwave power, I_0 the unsaturated amplitude and $P_{1/2}$ the microwave power at half saturation.

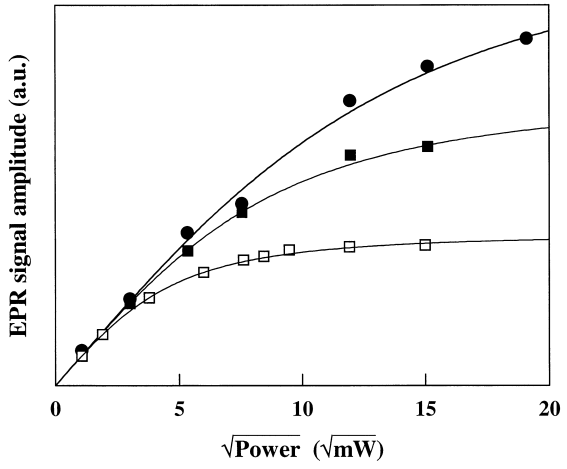

Fig. 4. Microwave power saturation of the S_0 (●) and S_2 state (■, □) EPR signals at 7 K. The curves have been fitted to Eq. 1 and normalized to the same initial slope. The parameter b of Eq. 1 was set free in the fits, resulting in $b = 1.14$ for all samples. Microwave power at half saturation of the S_0 signal in presence of 3% methanol (●) is $P_{1/2} = 250$ mW, of the S_2 multiline with 3% methanol (■) is $P_{1/2} = 85$ mW, and of the S_2 multiline without methanol (□) is $P_{1/2} = 27$ mW. EPR conditions: microwave frequency, 9.47 GHz; modulation amplitude, 10 G; modulation frequency, 100 kHz.

The exponent b is the so-called inhomogeneity parameter, which takes on the value one under conditions of exclusive inhomogeneous broadening of the signal [36].

The data and fits of Fig. 4 have been normalized to the same initial slope (I_0 of Eq. 1) to facilitate comparison of their saturation behavior, as expressed by the single parameter $P_{1/2}$. The fits of the data in Fig. 4 represent $P_{1/2}$-values of 250 ± 30 mW for the S_0 signal, 85 ± 10 mW for the S_2 multiline with 3% methanol, and 27 ± 5 mW for the S_2 multiline with no methanol present, all at 7 K. The S_0 EPR signal is a very fast relaxer, and cannot be saturated by the 365 mW maximum microwave power of our spectrometer. Consequently, our error in this determination is quite big. The S_2 multiline EPR signal also relaxes fast in the presence of methanol, but does approach saturation with the available microwave power. With no methanol in the sample, the S_2 multiline is more readily saturated than with methanol present. Since the S_0 signal is observable only in the presence of methanol, it is not clear whether this effect has an equivalent in the S_0 state. Thus methanol influences strongly both the spectral shape and amplitude and the microwave power relaxation of the EPR signals from the Mn cluster.

In the presence of 5% ethanol (which, like methanol, converts the $g = 4.1$ signal to the multiline signal [27,37]), the S_2 multiline signal relaxes with $P_{1/2} = 19 \pm 3$ mW (data not shown). This is close to the $P_{1/2}$-value of the alcohol-free sample, and very far from the dramatic increase observed upon the addition of methanol. The relaxation effect on the S_2 multiline signal thus seems methanol-specific, as is the appearance of the S_0 signal. It should also be

noted that it does not make any difference whether the S_2 sample is prepared by one flash at room temperature or by continuous illumination at 200 K (data not shown).

The microwave power saturation of SII_{slow} has been shown to oscillate with the S cycle in oxygen-evolving PSII preparations [19]. Signal II_{slow} relaxes faster in S_0 than in the S_1 state and it is an even faster relaxer in S_2 and S_3 at temperatures around 15–20 K [19,26]. The earlier experiments [19,26] were performed in the absence of methanol. We therefore found it interesting to compare the effects of methanol on SII_{slow} relaxation in the different S states. This could also cast light on whether methanol increases the relaxation rate of the S_0 signal as it does the S_2 multiline (Fig. 4). Fig. 5 shows $P_{1/2}$ of SII_{slow} in samples in the presence of 3% methanol as a function of the S state. The values are deconvoluted from data from flashed samples (as described in Section 2, see also [19]) to represent the separate S states. The $P_{1/2}$ values at 16.5 K displayed in Fig. 5 in the presence of 3% methanol are: S_1, 60; S_2, 130; S_3, 190; and S_0, 140 μW. In the absence of methanol we find substantially higher $P_{1/2}$ values in the studied S states; 350 μW in S_0, 700 μW in S_2 and 240 μW in S_1 (not shown) which are very similar to the values published earlier for the active state of PSII which were also measured in the absence of methanol [19,38]. Consequently, it seems that methanol makes the Mn cluster a faster relaxer (at least in the S_2 state) while it makes SII_{slow} a slower relaxer.

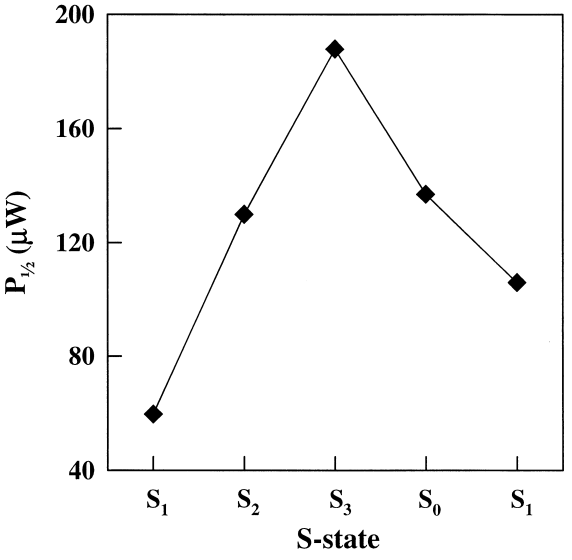

Fig. 5. Microwave power at half saturation ($P_{1/2}$) of Signal II_{slow} at 16.5 K in samples containing 3% methanol as a function of S state. The $P_{1/2}$ values are calculated by the deconvolution of saturation data from samples given 0–4 flashes, taking into account the S state distribution (see Section 2).

4. Discussion

We have presented a detailed study on the methanol concentration dependence of the S_0 and S_2 EPR signals. The $[MeOH]_{1/2}$ values of changes in the S_0 state EPR signal and the S_2 state multiline and $g=4.1$ signals were found to be close to 0.2% ($\approx$50 mM) in all three signals, indicating a similar action of methanol on the magnetic properties of the Mn cluster in the S_0 and S_2 states. The methanol concentration dependence of the S_2 multiline and $g=4.1$ signals were recently investigated [31]. For the $g=4.1$ signal suppression the result in [31] (54 mM $\approx$0.22%) is similar to ours, but there seems to be a difference for the S_2 multiline enhancement (23 mM $\approx$0.09% in [31]). However, as pointed out by Force et al. [31], their estimation was based on very few data points leading to low precision in the numbers. Our results are based on more data points and we conclude that methanol binds with similar affinity in both the S_0 and S_2 states.

Interesting questions are then, where is the site for methanol binding, and what is the nature of the methanol effect? We see two possibilities: (1) methanol binds directly to the Mn cluster, or it is positioned close enough to directly alter the magnetic interaction between the metal ions; or (2) methanol binds further away from the cluster, and causes longer range conformational protein changes that indirectly influence the magnetic properties of the cluster.

There exist indications that methanol might directly influence the Mn atoms. In a synthetic mixed valance Mn(III)–Mn(IV) complex it was shown that methanol can replace water to ligate directly to the Mn(III) ion [39] and it can be invoked that a similar condition might hold also for PSII. Indeed, Force et al. [31], from recent ESEEM measurement in the S_2 state have calculated that the distance between Mn and alcohol deuterons is 2.9–4.1 Å (dependent on the simulation method). These results are consistent with direct ligation of small alcohols (methanol and etha-

nol) to the Mn cluster in the S_2 state [31]. 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 both S states. In contrast, Boussac et al., who studied the S_0 state by ESEEM spectroscopy in presence of deuterated methanol [40], suggested that the observed changes were due to a global solvent effect on the protein structure rather than specific methanol binding to the Mn cluster. Thus, it is not entirely clear whether methanol binds directly to the Mn cluster in the S_0 state and further studies are needed to unambiguously clarify this.

If methanol binds to the Mn cluster, how can methanol induce the appearance of the $S = {}^1/_2$ S_0 signal and how can small alcohols (methanol and ethanol) affect the magnetic properties of the Mn cluster? It is likely that the appearance of the S_0 signal is due to a change of the magnetic couplings in the Mn cluster, resulting in a conversion from a higher spin state of the Mn cluster with non-resolved resonances in the high g-range of the spectrum. The effect of methanol would then be to increase the exchange coupling between the Mn ions, stabilizing the $S = {}^1/_2$ state. The protonation state of the oxo-bridges between the Mn atoms is not known, but a deprotonation of a bridge would induce such a change. The $|J|$-coupling is indeed increased by methanol in the S_1 and S_2 states (reviewed briefly in [23]), explaining the interconversion between the S_2 excited state $g = 4.1$ and multiline signals [28]. This conversion has recently been shown to include an intermediate $S = {}^5/_2$ state, that when trapped gives rise to EPR signals at $g = 6$ and $g = 10$ [41]. Population of such states can at present not be excluded for the S_0 state. Unlike the appearance of the S_0 signal the interconversion of the S_2 state signals can be induced by ethanol as well as methanol indicating that the two alcohols might interact through different mechanisms with Mn cluster.

Methanol also increases the relaxation rate of the S_2 multiline signal making it more difficult to saturate. This again shows that methanol interacts very closely with the Mn cluster in the S_2 state (compare [31]). A plausible explanation is that the stronger $|J|$-coupling makes the relaxation within the cluster more efficient. Interestingly, ethanol does not induce this change again implying that the two alcohols interact differently with Mn cluster. Thus, we can conclude that the appearance of the S_0 EPR signal and the relaxation effects on the S_2 multiline signal due to presence of methanol do not necessarily arise from the same binding phenomenon as the interconversion between the S_2 multiline and $g = 4.1$ signals, which can be induced by ethanol as well [27,37].

Our microwave saturation studies of SII_{slow} (Fig. 5) show that the relaxation enhancement of SII_{slow} by the Mn cluster which varies with the S states [19], is much affected by the presence of methanol. Without alcohol addition to the samples, we get similar $P_{1/2}$ values to those reported earlier [19]. At 20 K, it was found that Y_D^{ox} was a slow relaxer in S_1, a faster relaxer in S_0 and a much faster relaxer in the S_2 and S_3 states. The relaxation of Y_D^{ox} was found to be very temperature dependent, but it was similar in the S_2 and S_3 states at all measured temperatures. Here, we have measured $P_{1/2}$ of SII_{slow} at 16.5 K in the presence of methanol. In the S_1 state, $P_{1/2}$ is very low (60 μW) which is similar to what was earlier reported for Tris-washed PSII membranes [19]. Tris-washing removes the Mn cluster and it was consequently proposed [19], that the slow relaxation of SII_{slow} reflected that intrinsic relaxation of Y_D^{ox} (potentially influenced by other paramagnetic components in PSII). Thus the slow relaxation of SII_{slow} in the presence of methanol seems to decouple Y_D^{ox} magnetically from the Mn cluster, at least in the S_1 state. The other S states display higher $P_{1/2}$ values than the S_1 state and there is variation between the S states (Fig. 5). This oscillating behavior is similar to that observed in the absence of methanol, but the differences are not as large and also somewhat different in nature. While SII_{slow} relaxes faster in S_2 than in S_0 (at 20 K [19]) without additions, it appears to relax equally fast in the two states with methanol present (Fig. 5). In the presence of methanol, SII_{slow} also relaxes faster in S_3 than S_2 while it relaxed similarly in these two states in the absence of methanol [19]. Thus methanol clearly affects the magnetic coupling between the Mn cluster and Y_D^{ox}. Our present data are obtained with standard cw EPR which make them difficult to interpret fully. A further description of these interesting S state-dependent phenomena induced by methanol will involve direct T_1 measurements of Y_D^{ox} in different S states.

Fig. 3A displays a discrepancy in the $[MeOH]_{1/2}$ in the S_0 samples containing PPBQ/DMSO and S_0 sam-

ples without PPBQ/DMSO. This was unexpected and the difference in $[MeOH]_{1/2}$ indicates an effect of either PPBQ or the solvent, DMSO on methanol binding. This was investigated by studies on the S_2 multiline signal and our results indicate that it is the presence of DMSO (1%) rather than PPBQ that changes the $[MeOH]_{1/2}$ in the S_0 and S_2 multiline signals. This is different from studies of SII_{slow} relaxation where PPBQ was found to have an effect on the WOC [38], while there was no effect of the solvent DMSO alone. The reason for this difference is not known and PPBQ and DMSO will most likely interact in totally unrelated reactions with the Mn cluster.

High concentrations of DMSO (40–50%) have been shown to have an effect on the kinetics of the primary charge separation and stabilization in RC preparations of purple bacteria and PSII [42,43]. Related to this, Rubin and coworkers suggested that the effect of DMSO is due to the alteration of the H-bond state in the RC structure [42]. If Rubin's conclusions are applicable to the more exposed Mn cluster, also low concentrations of DMSO might have similar effects. Our results may then indicate that the methanol-sensitivity of the magnetic properties of the Mn cluster is related to the H-bond state of the protein matrix in the close vicinity of the Mn cluster. It is worthwhile to point out that there is very limited information on H-bonding to oxo-bridges or substrate in the Mn cluster. Consequently the subtle DMSO effect observed here will be pursued in future experimentation.

5. Conclusions

The addition of methanol results in significant modifications of the EPR signals from the S_0 and S_2 states of the WOC of PSII. The amplitudes of the hyperfine peaks of the S_0 and S_2 signals increase with the methanol concentration in a similar way. The amplitude of the S_2 excited state $g=4.1$ signal shows a concomitant decrease with increasing methanol concentration. The S_2 multiline signal relaxes faster in the presence of methanol, while ethanol does not affect the relaxation rate. Microwave power saturation measurements on SII_{slow} in the presence of methanol results in an S state-dependent oscillation of the $P_{1/2}$ values, similar to that measured without addition, but with lower values throughout. Thus, the magnetic coupling between the Mn cluster and Y_D^{ox} is much weakened by the presence of methanol.

From these results, we conclude that the methanol binding is similar in the S_0 and S_2 states. In order to establish the exact mechanism of the effect of methanol on the magnetic properties of the Mn cluster, and to explain the differences between ethanol- and methanol-treated samples, further investigations are required.

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