Analysis of the Interaction of Water with the Manganese Cluster of Photosystem II Using Isotopically Labeled Water†

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Received August 12, 1996; Revised Manuscript Received October 30, 1996⊗

ABSTRACT: The association of water with the Mn of the water oxidizing complex was investigated using $H_2{}^{17}O$- and ^2H_2O-reconstituted lyophilized photosystem II particles. The pulsed electron paramagnetic resonance (EPR) technique of electron spin echo envelope modulation (ESEEM) was used to investigate the interaction of the magnetic ^2H and ^{17}O nuclei with the paramagnetic S_2 state of the Mn complex and other photosystem II components. ESEEM offers a much more specific and sensitive detection of this type of interaction than continuous wave (CW) EPR. Unlike earlier reports using CW EPR, these experiments did not detect any interaction of water with the multiline EPR signal from the S_2 state of the Mn complex. No signals indicating specific interaction of either H or O with the multiline signal were detected. Signals due to ^2H and ^{17}O were detected only at the Larmour frequency, indicating nonspecific "distant ENDOR" effects. A weak interaction with ^{17}O was detected both in S_1, when the Mn is EPR silent, and in S_2, but only on the high-field side of $g = 2$. This interaction may be with the Rieske iron−sulfur center in the cytochrome b_6f complex. The results were the same whether the multiline signal was generated by 200 K illumination of dark-frozen samples, or by room temperature illumination in the presence of the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Illumination at room temperature in the presence of an electron acceptor to allow multiple turnovers of the system with cycling of the S states did not result in the appearance of any new interactions. These results appear to exclude close (less than 6 Å) binding of water to the Mn center giving rise to the multiline signal, and also to exclude mechanisms in which water oxidation involves the breaking and re-formation of the μ-oxo bridges of the Mn complex. They cannot, however, exclude models in which water binding to the manganese complex and direct oxidation by the manganese complex occur in the higher S states, or are catalyzed by one bis(μ-oxo) Mn dimer while oxidizing equivalents are accumulated in the S_2 state by a second bis-(μ-oxo) Mn dimer.

The oxidation of water by plants, algae, and cyanobacteria provides the reductant for photosynthetic carbon fixation and is the source of atmospheric oxygen. Water oxidation is catalyzed by a membrane-bound pigment protein complex, photosystem II, containing a Mn complex essential for water oxidation (Debus, 1992; Nugent, 1996).

Photochemical charge separation in photosystem II (PS II)[1] is a one-electron process while water oxidation is a concerted process involving four electrons. Joliot (Joliot et al., 1969) and Kok (Kok et al., 1970) showed that this could be described by a cyclic process involving five redox states, termed S_{0-4}, and that the presence of a number of metastable redox states could be demonstrated in chloroplasts following flash illumination. Under appropriate experimental conditions, dark-adapted chloroplasts exposed to short actinic flashes of light evolve oxygen on the third flash, and subsequently every fourth flash. If S_0 is the most reduced and S_4 the most oxidized S state, these results suggest that the dark-stable state is S_1. Many experiments have in fact shown that when the S states are randomized by continuous illumination and then transferred to the dark, the complex contains 25% S_0 and 75% S_1 after a few minutes, converting slowly to 100% S_1 over a period of hours (Vermaas et al., 1984). The chemical identity of the S states and the chemistry of water oxidation are still uncertain. However, it is clear that Mn is involved in the complex and that Mn redox changes occur during at least some of the S state changes [Zimmermann & Rutherford, 1984; see Debus (1992) for a review].

Structural information about the Mn complex and the redox states of the Mn has come from X-ray spectroscopy and comparison with synthetic model compounds (Baldwin et al., 1994; Bossek et al., 1990; Horwitz et al., 1993; Kirby et al., 1981b; Randall et al., 1995). These measurements are concentrated on the S_1 and S_2 states, experimentally the most accessible. There is general agreement that the Mn complex consists of bis(μ-oxo)-bridged Mn dimers, and that the complex probably contains two such dimers in different environments (MacLachlan et al., 1994a; Yachandra et al., 1993). The redox state of the Mn is probably $Mn(III)_2Mn(IV)_2$ in S_1 and $Mn(III)Mn(IV)_3$ in S_2 (Kirby et al., 1981a; Kusunoki et al., 1990; MacLachlan et al., 1994b; Penner-Hahn et al., 1990; Turconi et al., 1995; Yachandra et al., 1986). The S_2 state is paramagnetic and has two interconvertible EPR signals, a $g = 4.2$ signal (Casey & Sauer, 1984; de Paula et al., 1986) and a complex "multiline" signal with about 18 lines around $g = 2.00$ (Dismukes & Siderer, 1981),

† This work was supported by BBSRC Grant GR/H93330.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: CW, continuous wave; DCBQ, 2,6-dimethyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; EXAFS, extended X-ray absorption fine structure; PS II, photosystem II; XANES, X-ray absorption near-edge structure.

S0006-2960(96)02010-7 CCC: $14.00

which probably originate from different configurations (Cole et al., 1987; de Paula et al., 1986; Zimmermann & Rutherford, 1986) or different valence distributions of the complex (Boussac et al., 1996).

Current models of water oxidation are largely based on redox cycling of the Mn complex as a charge accumulator. Many suggest that the Mn complex is also the site of water binding and oxidation. Possible mechanisms of water oxidation which have been suggested involve peroxide formation either external to the bis(μ-oxo) bridges [see Wydrzynski et al. (1996)] or involving the oxygens of the bis(μ-oxo) bridges, with breaking and re-formation of the μ-oxo bridged dimers [see Wang et al. (1994) and references cited therein]. Recent studies using mass spectrometry have indicated that water binding to the water oxidation complex occurs in two phases (Messinger et al., 1995). At S_3, one substrate water molecule exchanges slowly ($t_{1/2} \approx 500$ ms), the other quickly. The presence of the slowly exchanging substrate water molecule implies binding prior to the formation of S_3. These experiments are indirect and do not identify the binding site. A more direct approach investigating the binding of isotopically labeled water or water analogues to the Mn complex in the S_2 state by measuring changes in the properties of the multiline EPR spectrum has been taken by a number of workers.

NH_3 is an analogue of water which inhibits oxygen evolution by the water oxidizing complex. CW EPR studies showed that NH_3 binding to the complex results in changes in the multiline signal (Beck et al., 1986). More specific information about this effect was obtained using ESEEM spectroscopy which clearly showed that NH_3 treatment results in the appearance of N modulation of the ESEEM signal at 3500 G in the high-field part of the multiline signal (Britt et al., 1989). An amido bridge between metal ions was proposed as the source of the modulation.

Interaction of isotopically labeled water with the multiline signal has been investigated in two CW EPR studies, one using $H_2{}^{17}O$ (Hansson et al., 1986) and one ^2H_2O (Nugent, 1987). In both these studies, weak interaction between the magnetic nucleus and the multiline signal was reported.

In the ^2H_2O experiments, the observed effect was an increased resolution of the fine structure and small changes in the fine structure of the hyperfine lines in the spectrum formed by illumination at 283 and 200 K. These changes indicate the presence of ^2H in the environment of the manganese cluster; they do not specifically demonstrate water binding as exchange of protein protons in the Mn binding region could produce the same effect.

In the ^{17}O experiments, the observed effect was a small overall line broadening, observed essentially as a small loss of resolution in the spectrum. No peak to peak width changes were observed. The hyperfine coupling constant for ^{17}O was 0.5 mT or less, excluding the possibility of incorporation of ^{17}O into bis(μ-oxo) bridges or the formation of superoxide or hydroxyl radicals, but not of peroxides.

In view of the lack of specificity in the reported CW EPR experiments, and the importance of understanding the interaction between water and the Mn complex in understanding the mechanism of photosynthetic water oxidation, we have used ESEEM spectroscopy to investigate the interaction of $H_2{}^{17}O$ and ^2H_2O with the S_2 multiline signal. ESEEM spectroscopy offers the possibility of a much more precise demonstration of the interaction of the magnetic nuclei with the Mn complex and of obtaining information about the nature of the interaction.

EXPERIMENTAL PROCEDURES

Sample Preparation. Oxygen-evolving PS II subchloroplast membranes ("BBY" particles) were prepared by the method of Berthold et al. (1981) with the modifications of Ford and Evans (1983). Samples for lyophilization were suspended in a buffer containing 20 mM MES−NaOH, 15 mM NaCl, 5 mM $MgCl_2$, and 0.5 M sucrose, pH 6.3 (buffer A). Additionally, for separate experiments, BBY particles were lyophilized from buffer A without any salt added (20 mM MES−NaOH and 0.5 M sucrose, pH 6.3, termed "no salt") or from buffer A + 10 mM $CaCl_2$ ("+Ca^{2+}").

Samples for EXAFS were prepared by washing the PS II particles in 40 mM MES−NaOH, 10 mM NaCl, and 1 mM EDTA, pH 6.0, followed by centrifugation at 40000*g* for 10 min. The pellet was resuspended in buffer A (standard, "no salt" or "+Ca^{2+}") and lyophilized. The resulting material was powdered and loaded into polycarbonate holders.

Samples for continuous wave EPR were reconstituted with distilled water to 10 mg of Chl/mL and loaded into calibrated 3 mm quartz EPR tubes.

For the pulsed EPR experiments, lyophilized BBY particles were resuspended in H_2O, ^2H_2O (99.9%, Aldrich), or $H_2{}^{17}O$ (50% enriched, Monsanto Research Corp.) to approximately 10 mg of Chl/mL and transferred to EPR quartz tubes. The samples were dark-adapted for more than 1 h on ice to allow equilibration in the S_1 state, and frozen in liquid nitrogen after the addition of 0.5 mM DCBQ. The S_2 state was achieved by continuous illumination of the samples at (a) 200 K in an ethanol/solid CO_2 bath for 15 min or at (b) 273 K for 90 s following addition of 0.5 mM DCMU.

Sample Characterization. The oxygen evolution rates of lyophilized PS II particles resuspended in H_2O, ^2H_2O or $H_2{}^{17}O$ were determined using a Clark-type electrode at 298 K, in a H_2O buffer containing 20 mM MES−NaOH, pH 6.0, 10 mM NaCl, and 25 mM $CaCl_2$. Ferricyanide and DCBQ (0.5 mM) were added as electron acceptors.

Continuous wave EPR spectra were recorded on a JEOL RE1X spectrometer with an Oxford Instruments liquid helium cryostat.

Fluorescence EXAFS measurements were carried out at the CLRC Daresbury Laboratory, U.K., on Station 8.1 with a slitless Si(111) or Si(220) double-crystal monochromator and a 13-element Ge solid-state detector. Spectra were recorded at 77 K with a beam energy of 2 GeV and an average beam current of 160 mA. Each scan lasted approximately 1 h. Five scans were collected per sample. The edge energies, defined as the first inflection point on the rising absorption edge, were determined from the second derivative of smoothed experimental spectra. Data analysis was performed on the raw EXAFS in *k* space with use of the Fourier transform confined to model building, as described previously (MacLachlan et al., 1992, 1994a). The quality of the fit was determined visually and by comparing the fit index, FI (Lytle et al., 1989), as described in MacLachlan et al. (1992, 1994a). For fitting purposes, the only light atoms coordinated to the manganese were considered to be oxygens. This is consistent with current evidence which suggest only a small number of nitrogen ligands (Andreasson, 1989; Britt et al., 1989). We adopted

a fitting procedure whereby the coordination numbers were varied systematically in steps of 1.0 while the Debye−Waller factors, $2\sigma^2$ where σ is the root mean square deviation in the interatomic distance, were refined to obtain the best simulation (MacLachlan et al., 1992, 1994a). From these calculations, the errors associated with the coordination numbers are ±20% for the short oxygen and manganese shells and ±30−50% for the longer oxygen shell. The estimated error for the radii is ≤2% and for the Debye-Waller factors ±30−50%.

ESEEM. Spectra were recorded on a Bruker ESP380E X-band pulsed spectrometer which was equipped with a variable Q dielectric resonator (Bruker Model 1052 DLQ-H 8907). The sample was maintained at 4 K by an Oxford Instruments CF395 flowing gas cryostat. For a 16 ns pulse in a $S = {}^1/_2$, $g_e = 2.002$ system, the maximum microwave magnetic field generated by a 1 kW travelling wave tube amplifier was approximately 6 G within the 10 mm homogeneous region of the resonator. All ESEEM spectra were acquired with a cavity factor (Q) of about 100 which corresponds to a minimum dead time of approximately 100 ns. Three-pulse 1D ESEEM spectra were acquired with the phase-cycled stimulated echo sequence ($\pi/2-\tau-\pi/2-T-\pi/2-$acq.) (Fauth et al., 1986) with $\pi/2 = 16$ ns where τ is held constant and T is varied. The echo was recorded with the Bruker integrator ESP380-IN1078 with an 80 ns integration gate width. For a typical sweep, T comprised 256 data points with an 8 ns increment and an initial value of 32 ns. All the spectra were recorded at 4 K and with a repetition time of 5 ms. Data analysis was performed using Bruker WINEPR software (Thiele et al., 1992).

RESULTS

Characterization. Upon resuspension in H_2O, ^2H_2O, or $H_2{}^{17}O$, the lyophilized photosystem II particles showed oxygen evolution rates at the native levels, i.e., within ±10% from the control (nonlyophilized) particles. Consistently, for five different lyophilization and reconstitution experiments, the PS II particles resuspended in ^2H_2O showed approximately 7−12% higher oxygen evolution rates than the respective control sample.

No adventitious Mn^{2+} was detected by EPR before or after the lyophilization. Figure 1 shows the CW EPR spectrum of lyophilized PS II particles, poised in the S_2 state after being reconstituted in water. These particles display a normal "multiline" signal, centered around $g = 2$ and consisting of approximately 18 lines.

The Mn K-edge positions for all the lyophilized BBY samples, whether or not salt was present, were 6551.0 ± 0.1 eV, which was similar to the native S_1 state, indicating that the water oxidation complex in the lyophilized preparation was in the S_1 state.

The Fourier-filtered, k^3-weighted EXAFS data and their Fourier transforms obtained for the lyophilized BBY particles are shown in Figure 2. In all cases, three distinct peaks at approximately 1.8 Å, 2.7 Å, and 3.6 Å were present in the Fourier transforms.

For the first peak in all our simulations of BBY lyophilized samples, a short ca. 1.86 ± 0.01 Å Mn−O distance is required. For simplicity, the first peak was modeled with only one shell of scatterers wherever the quality of the fit allowed. This shell can be very well described as oxygens forming μ-oxo bridging ligands. For particles lyophilized from a buffer containing Ca^{2+} (see Experimental Procedures), the first peak in the Fourier transform was satisfactorily modeled with parameters that are very similar to ones for native BBY particles as shown in Table 1 and that are in agreement with previous studies (DeRose et al., 1994; MacLachlan et al., 1992; Yachandra et al., 1986). However, for the particles lyophilized from buffers not containing Ca^{2+}, a much better fit was obtained by adding another shell of light atoms at 2.20 Å (see Table 1). The dominant contribution to the first peak comes from the μ-oxo bridging ligands (1.86 Å). It has been shown that an additional shell of light atoms is sometimes needed for a good fit to this peak, its distance varying from 1.95 Å (DeRose et al., 1994; MacLachlan et al., 1992, 1994a; Mukerji et al., 1994) to 2.1−2.3 Å (George et al., 1989; Guiles et al., 1990a,b; Hatch et al., 1995, Penner-Hahn et al., 1990).

The second peak can be best described with one Mn−Mn interaction (per Mn) at a distance of 2.70 ± 0.04 Å which, together with the short Mn−O distance of ≤1.9 Å, fits well

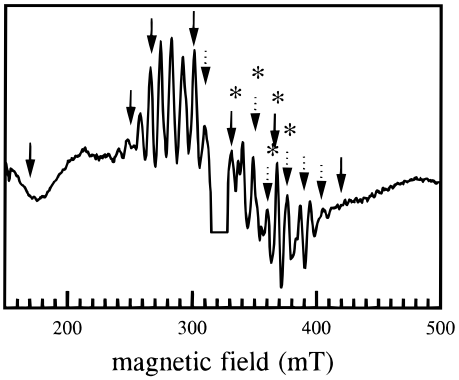

FIGURE 1: CW EPR spectrum of the "multiline" signal of PS II particles lyophilized from buffer A "+Ca^{2+}" and resuspended in distilled water. Spectrometer conditions: 10 mW microwave power; 1.6 mT modulation amplitude; microwave frequency 9.065 GHz; temperature 6 K. The central region of the spectrum has been erased to remove the $Y_D^\bullet$ radical signal. Arrows indicate magnetic field positions at which ESEEM experiments on the ^1H_2O- and $H_2{}^{17}O$-reconstituted BBY particles were performed. Stars mark the field values at which a modulation at 2 MHz was found in the $H_2{}^{17}O$-reconstituted PS II.

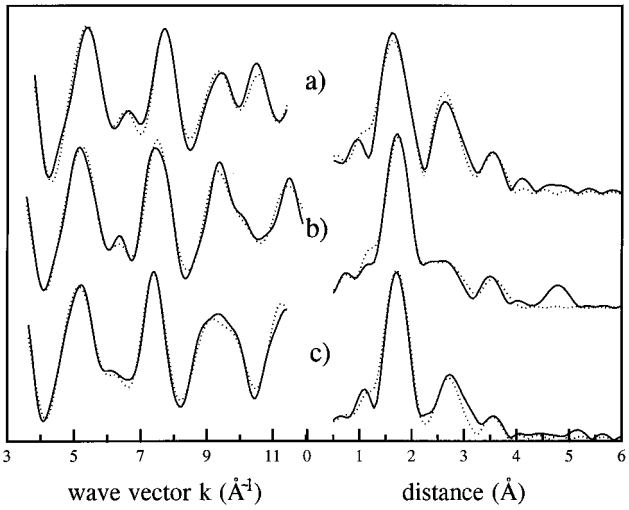

FIGURE 2: Fourier-filtered, k^3-weighted EXAFS data (left-hand side) and their Fourier transforms (right) for PS II particles lyophilized from different buffers. Top traces: reconstituted in buffer A "+Ca^{2+}". Middle: reconstituted in buffer A. Bottom: in "no salt" buffer. The solid line represents experimental data and the dotted line the modeled fit. For details of the fit parameters, see Table 1.

Table 1: EXAFS Fitting Parameters for PS II Particles Lyophilized from Different Buffers[a]

	"no salt"		buffer A			
parameter	(a)	(b)	(a)	(b)	"+Ca^{2+}"	S_1 BBY[b]
*r*1 (Å)	1.84	1.84	1.87	1.89	1.85	1.86
*r*2 (Å)		2.20		2.20		
*r*3 (Å)	2.70	2.70	2.72	2.74	2.69	2.70
*r*4 (Å)	3.61	3.61	3.70	3.74	3.63	3.69
α1	0.008	0.008	0.014	0.012	0.023	0.028
α2		0.023		0.023		
α3	0.009	0.008	0.018	0.019	0.007	0.004
α4	0.019	0.019	0.016	0.018	0.011	0.008
E_0	25.5	23.9	25.6	21.5	27.6	26.8
N_l	13.7	13.9	14.1	14.2	12.8	14.4
p	7	9	7	9	7	7
FI × 10^3	0.331	0.221	0.575	0.220	0.361	0.232
R	18.49	15.24	25.48	14.78	17.57	15.07
$\epsilon_\nu^2 \times 10^6$	4.52	4.19	7.58	4.00	5.62	2.92

[a] Raw data were Fourier-filtered in a 0.7−4.5 Å window. (a) and (b) represent fits of the same data set to two different models: (a) the first peak fitted with only one shell of light scatterers; (b) with two sub-shells. [b] Data from MacLachlan et al. (1992). Estimated errors are given in under Experimental Procedures.

with a model of bis(μ-oxo)-bridged Mn dimers (MacLachlan et al., 1992; Yachandra et al., 1986, 1993). It is apparent from Figure 2 that the EXAFS modulations and the Fourier transforms of the second peak for the BBY samples lyophilized from different buffers are different. There is a reduction in intensity of the 2.7 Å feature in the sample lyophilized from the "no salt" buffer compared to PS II lyophilized with 10 mM Ca^{2+}, and in the case of the particles lyophilized from buffer A (without Ca^{2+}), this peak is even weaker and poorly resolved (see Figure 2).

The third peak at a distance of 3.65 ± 0.07 Å can be modeled with Mn, Ca, or C (and a combination of these) without a significant difference in fit quality. This peak shows slight variations in intensity for different lyophilized PS II preparations, being the most intensive in the particles lyophilized in the presence of Ca^{2+}.

In summary, we find that the EXAFS properties of the BBY particles lyophilized from buffer A + 10 mM $CaCl_2$ ("+Ca^{2+}") are very similar to native BBY particles as characterized previously (MacLachlan et al., 1992). The PS II particles used for the pulsed EPR experiments described below were therefore lyophilized from buffer A + Ca^{2+}.

ESEEM. Three-pulse ESEEM spectra of photosystem II particles reconstituted in ^2H_2O were obtained at two field positions situated on either side of $g = 2$: 268.9 mT (g = 2.42) and 355.1 mT ($g = 1.95$). Figure 3 shows three-pulse ESEEM spectra of the S_1 and S_2 states at 355.1 mT and their difference. From the comparison of these with corresponding spectra of particles resuspended in ^1H_2O (see Figure 4), a complete absence of the peak at the proton Larmour frequency (approximately 15 MHz) is apparent. The ^2H_2O spectra are dominated instead by a single peak at the $^2H_2$ Larmour frequency, deriving from weak, distant ENDOR interactions of exchangeable protons. The fact that no detectable modulation is present at the ^1H Larmour frequency and that it has been completely replaced by ^2H proves that the reconstitution of lyophilized PS II particles with ^2H_2O has been complete. The peak at approximately 4.5 MHz represents the hyperfine coupling of nitrogen to Mn, as previously assigned (DeRose et al., 1991). They presented data at one field value. However, we find that this peak is

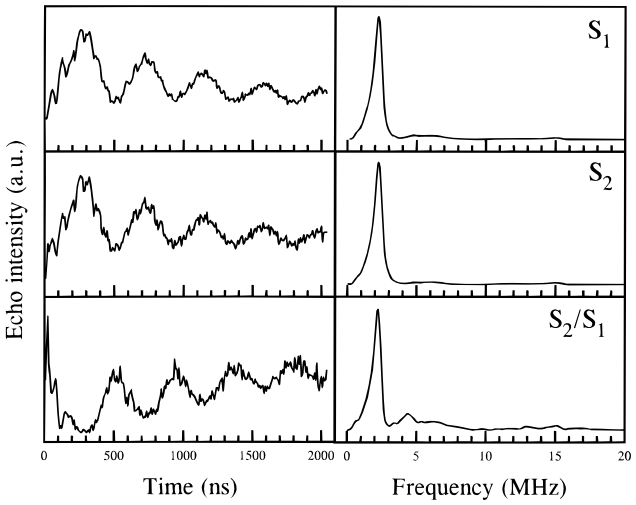

FIGURE 3: Time-domain three-pulse ESEEM spectra (left) and their Fourier transforms (right) of lyophilized PS II particles reconstituted in ^2H_2O in S_1 and S_2 states and the difference, S_2/S_1 (bottom panel). Spectrometer conditions: field position 355.1 mT; microwave frequency 9.71 GHz; τ = 112 ns; temperature 4 K.

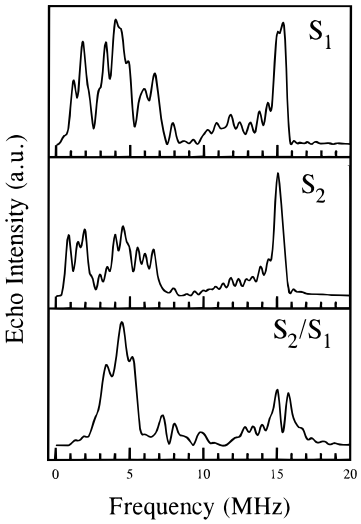

FIGURE 4: Fourier-transformed three-pulse ESEEM spectra of lyophilized PS II, reconstituted in H_2O. Top panel: particles in the S_1 state. Middle: particles in the S_2 state. Bottom panel: their difference, S_2/S_1. Field position 355.1 mT; temperature 4 K. Spectrometer conditions as in Figure 3.

present in all the spectra and at all magnetic field values (see below), but for ^2H_2O-reconstituted PS II particles, the $^2H_2$ modulation strongly dominates the spectrum.

Three-pulse ESEEM spectra of the lyophilized PS II particles reconstituted in H_2O and $H_2{}^{17}O$ were recorded in both S_1 and S_2 states, at the following magnetic field positions: 450 mT ($g = 1.54$), 394.1 mT ($g = 1.76$), 355.1 mT ($g = 1.95$), 321.5 mT ($g = 2.16$), 286.9 mT ($g = 2.42$), 267.9 mT ($g = 2.59$), and 182.2 mT ($g = 3.81$). These values were chosen to cover the full range of the "multiline" signal of the manganese cluster in the S_2 state, as shown in Figure 1 (solid arrows). All the spectra were analyzed individually, and, subsequently, in order to detect any contributions to the echo envelope from the ^{17}O nucleus, difference spectra were produced. The difference spectra were obtained by division of the original spectra in the time domain and then Fourier-transforming the resulting difference.

Three-pulse ESEEM spectra at 355.1 mT of lyophilized PS II reconstituted in H_2O are shown in Figure 4. The

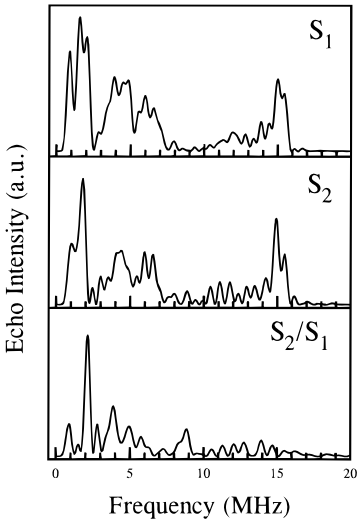

FIGURE 5: Fourier-transformed three-pulse ESEEM spectra of lyophilized PS II particles reconstituted in $H_2{}^{17}O$. From top to bottom: S_1 and S_2 states and their difference, S_2/S_1. Field position 355.1 mT; temperature 4 K. Other spectrometer conditions as in Figure 3.

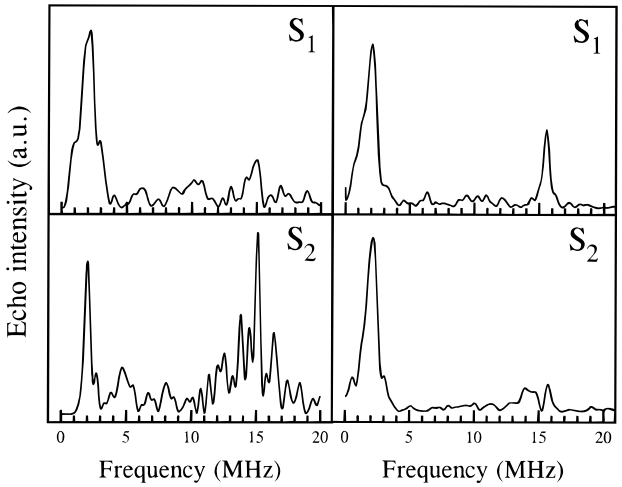

FIGURE 6: $H_2{}^{17}O/H_2O$ difference spectra of lyophilized PS II particles in S_1 and S_2 states. The original spectra (shown in Figures 4 and 5) were divided in the time domain, and the result was Fourier-transformed. Left-hand side: spectra of a sample in which the S_2 state was achieved by illumination at 200 K. Right-hand side: the S_2 state was achieved by illumination at 273 K upon addition of DCMU. Field position 355.1 mT. Other spectrometer conditions as in Figure 3.

spectra are of particles poised in S_1 and S_2 states and their difference (S_2/S_1). Analogous spectra for particles resuspended in $H_2{}^{17}O$ are shown in Figure 5. The most obvious difference between these two sets of spectra is the presence of the modulation around 2.0 MHz in the PS II particles resuspended in $H_2{}^{17}O$. The Larmour frequency of the ^{17}O nucleus is 2.05 at this field. This difference is more clearly visible in the $H_2{}^{17}O/H_2O$ difference spectra of both S_1 and S_2, shown in Figure 6. The modulation at approximately 2 MHz is present in both states, and there is no apparent difference between S_1 and S_2 spectra of lyophilized PS II reconstituted in $H_2{}^{17}O$. Moreover, this modulation is seen only at two of the field positions investigated: 355.1 mT and 394.1 mT. This indicates an interaction not originating from the multiline signal. Furthermore, this means that water is not binding or interacting closely (<6 Å) with the paramagnetic manganese in the S_2 state.

To investigate the possibility that water oxygen is being incorporated in the bis(μ-oxo) bridges at a later stage in the water oxidation process, the samples were thawed and illuminated for 2 min with a 75 W light at room temperature in the presence of the electron acceptor DCBQ, to allow for S-state turnover. The samples were then dark-adapted to restore the S_1 state (1.5 h, at 0 °C) and frozen in the dark. The S_2 state was then generated again by a 200 K illumination, and the ESEEM spectra were recorded as described above. The ESEEM spectra of the PS II particles resuspended in H_2O, ^2H_2O, or $H_2{}^{17}O$ after the turnover treatment did not show any new features or significant differences as compared to the spectra prior to the turnover treatment. This excludes the possibility that oxygen from water is assimilated into the bis(μ-oxo) bridges, or other components in the vicinity of the EPR-active manganese during the water oxidation reaction.

The presence of the modulation at 2 MHz at the high-field side of $g = 2$ was investigated further in $H_2{}^{17}O$ reconstituted samples, by collecting ESEEM spectra at narrowly spaced field positions at the high-field side of $g = 2$. The field values for these additional experiments are indicated by dotted arrows in Figure 1. The field positions at which a modulation from ^{17}O was present are marked by a star. It is evident from the distribution of these "starred arrows" in Figure 1 that the presence of modulation coincided with the expected spectral range of EPR signals from the iron−quinone electron acceptors (Q_A, Q_B) or of the Rieske iron−sulfur center. The Rieske center is present in the preparation as the BBY preparation from spinach contains a significant amount of the cytochrome b_6f complex, while $Q_A^{\bullet-}$ is present only in the S_2 state.

The results of the experiments described seem to indicate that, in the S_2 state, oxygen is not interacting with the manganese species responsible for the multiline signal. The sole new contribution from the ^{17}O nucleus is a modulation at its Larmour frequency, which may arise from a weak interaction of water ($H_2{}^{17}O$) with the Q_B site or with the Rieske iron−sulfur center.

In an attempt to clarify the origin of the signal displaying the 2 MHz modulation, DCMU was added to a set of samples resuspended in H_2O, ^2H_2O, or $H_2{}^{17}O$, and the three-pulse ESEEM experiments in the S_1 and S_2 states were repeated. The S_2 state was induced by illumination at 273 K (see Experimental Procedures). Figure 6 shows the S_2/S_1 difference spectra obtained for the $H_2{}^{17}O$ samples. The presence of a modulation at 2 MHz is evident, and the spectra are overall very similar to those obtained without DCMU. This would seem to exclude Q_B as the interacting species. The Rieske center is therefore most likely to be the interacting species.

DISCUSSION

The validity of these experiments depends on the success of the lyophilization procedure in retaining the native structure and activity of the water oxidizing complex. This has been confirmed by a wide range of assay procedures, including both activity measurements and spectroscopic analysis. The rate of oxygen evolution by the lyophilized samples is within ±10% of the rate of the samples before lyophilization, in agreement with a recent report (Karge et al., 1996). Samples resuspended in ^2H_2O consistently showed a small increase in activity over other samples. This

is most probably due to increased thermal stability of the PS II particles (Renger et al., 1994). The rate of oxygen evolution itself is not a conclusive proof of sample intactness since the assay buffer contains the cofactors needed to restore normal function (Ca^{2+}, Cl^-, electron donors and acceptors). Therefore, we characterized the lyophilized PS II by CW EPR and EXAFS. The CW EPR spectrum of the S_2 multiline signal was indistinguishable from that in the starting material and showed no major changes resulting from the isotopic composition of the resuspending water. The material appeared to be in the S_1 state. After resuspension in water in the dark and freezing in the dark, the lyophilized samples were converted to S_2 with the normal multiline signal by 200 K illumination. XANES of the lyophilized material showed the edge position was 6551.0 ± 0.1 eV. This is very similar to the native S_1 edge position in our experiments (MacLachlan et al., 1992; Turconi et al., 1995). These results are in contrast to experiments on lyophilized material by Kawamoto and Asada (1990), who observed a two-flash delay in oxygen evolution measurements on previously lyophilized material. This would suggest the material was in an S_{-1} state. We have made parallel experiments on lyophilized core particles made with heptyl thioglucoside in which both the donor and acceptor sides of PS II are modified (results not presented). These showed an edge position indicating a more reduced state of the Mn complex after lyophilization. However, the procedure used with the BBY samples in our experiments appears to maintain the material in the S_1 state. EXAFS of the lyophilized samples also showed an essentially unmodified structure for the Mn complex. This is particularly true for the BBY particles lyophilized from a Ca^{2+}-containing buffer. Lyophilization from a medium not containing Ca^{2+}or any salt resulted in modified EXAFS, affecting mostly the first and the second shell and indicating a changed manganese environment, possibly due to altered electrostatic interactions. Although high-quality spectra were obtained from compressed dried samples, there was no significant advantage in the signal to noise ratio compared to the most concentrated frozen paste samples. Taken together, these analyses show that the native structure and function of the water oxidation complex is maintained during the lyophilization and rehydration processes.

The field-swept pulsed EPR spectra of the S_1 samples show a large signal around $g = 2.00$, as has been reported (Britt et al., 1989, 1992; Zimmermann et al., 1993). The field position of this spectrum and comparison with the conventional CW EPR spectrum of the samples suggest that this signal arises in part from Cu and the tyrosine D (signal II), and may also include contributions from Q_B, oxidized cytochromes, and the Rieske center. The presence of this signal underlying the S_2 signal makes interpretation of the results more difficult. In all the samples containing ^1H_2O, there is a large signal at 14.9 MHz, the proton Larmour frequency due to "distant ENDOR", with the interaction of one or more of the components contributing to the signal with the protons of the medium. In samples in ^2H_2O, this signal is replaced by a signal at 2.3 MHz, the Larmour frequency of ^2H. This result shows clearly that the replacement of ^1H is very effective as no 14.9 MHz signal is seen in the lyophilized samples resuspended in ^2H_2O. This signal was seen at both the high-field and the low-field position investigated. Comparison of the spectra in S_1 and S_2 either by simple subtraction or by division of the time domain data prior to Fourier transformation shows that no new signal appears in the S_2 state. There is no indication of the close association of ^2H with the Mn complex giving rise to the signal, either through hydrogen bonds or through other interaction. Indeed, the results seem to suggest that there is no water or exchangeable protons close enough to the center to even give rise to nonspecific "distant ENDOR" effects. Because of the nature of the experiment, in which changes in the redox state of other electron transfer components during illumination may affect the interacting species seen in the dark base line, it is not possible to draw quantitative conclusions about the concentration of centers giving rise to the observed interactions. It is therefore not possible to decide if there is "distant ENDOR" modulation of the S_2 signal by protons. However, it is clear from the results that no new interactions are seen between S_2 and the hydrogen of water.

In the experiment with ^{17}O, there is a small signal in the ESEEM spectra of S_1 or S_2. Division of the time domain data for $^{17}O/^{16}O$ reveals the presence of a signal at 2 MHz. However, this signal is present in both S_1 and S_2 samples, and is unaltered whether the S_2 signal is formed at low temperature or at room temperature, or after cycling of the S states. Analysis of the field range of this signal indicated that it was only detectable on the high-field part of the spectrum (355−410 mT, $g = 1.95-1.70$). Consequently, we conclude that this signal does not arise from interaction of the water with the Mn center. The spectral range of the signal suggests it may arise from Q_B or the Rieske center. These sites are expected to be accessible to the aqueous environment. The signal was not removed with DCMU, which would be expected to displace Q_B from its binding site. The identity of the component weakly interacting with ^{17}O is therefore uncertain, with the Rieske iron−sulfur center of the cytochrome b_6f complex the most likely candidate.

The results indicate that there is no direct interaction or binding of water to the Mn complex giving rise to the S_2 multiline signal. This conclusion is in contrast to those drawn from CW EPR experiments. However, the spectral changes seen in those experiments were very small, compared to the line broadening seen for ^{17}O-coordinated Mn reported earlier (Eads et al., 1988; Reed & Leyh, 1980; Wittinghofer et al., 1982). At this point, we must note that the nuclear spin of ^{17}O, $I = {}^5/_2$, could lead to significant quadrupole effects in ESEEM, making it difficult to detect. However, ^{17}O ESEEM has been reported for the Fe center in Cyt P450 (Thomann et al., 1995). Moreover, upon releasing the manganese from the water-oxidation complex by heat treatment (10 min at 60 °C) in the same sample, we were able to detect modulation at the ^{17}O Larmour frequency on the six-line hexaaquo-Mn^{2+} signal at magnetic field positions on both sides of $g = 2$.

The $^{17}O/^{16}O$ results are also different from those found using NH_3 as a water analogue (Britt et al., 1989), when N modulation of the signal at 350 mT was clearly observed. The effect of NH_3 binding to the Mn cluster is, however, readily observable on the CW EPR multiline signal, which is drastically changed (Beck et al., 1986). This may indicate that the NH_3 binding site or mode is different than that of water. Further investigation of the factors controlling NH_3 binding may be of interest.

Although the results show no interaction with the multiline signal, they do not exclude water binding to the water oxidation complex in S_2. Binding may occur at this stage

at a site distant from the Mn complex, implying that the oxidant accumulation and water binding sites are different. Although it has been suggested that the EPR signals arising from the S_2 state require magnetic coupling between two Mn dimers (de Paula et al., 1986; Kim et al., 1990, 1992; Kusunoki, 1992), the multiline signal can be simulated on the basis of a single Mn dimer (Åhrling & Pace, 1995). If the multiline signal may arise from only one bis(μ-oxo)-bridged Mn dimer, our results cannot exclude the possibility that water is bound by a second Mn dimer in a different environment at this stage in the S state cycle.

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BI962010B