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Structure of the Manganese Complex of Photosystem II upon Removal of the 33-Kilodalton Extrinsic Protein: An X-ray Absorption Spectroscopy Study[†]

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ABSTRACT: The structure of the Mn complex of photosystem II (PSII) was studied by X-ray absorption spectroscopy. Oxygen-evolving spinach PSII membranes containing 4-5 Mn/PSII were treated with 0.8 M $CaCl_2$ to extract the 33-, 24-, and 16-kilodalton (kDa) extrinsic membrane proteins. Mn was not released by this treatment, but subsequent incubation at low Cl^- concentration generated preparations containing 2 Mn/PSII. The Mn X-ray absorption K-edge spectrum of the $CaCl_2$-washed preparation containing 4 Mn/PSII is very similar to the spectrum of native PSII, indicating that the oxidation states and ligand symmetry of the Mn complex in these preparations are not significantly different. The Mn extended X-ray absorption fine structure (EXAFS) of $CaCl_2$-washed PSII fits to a Mn neighbor at ~2.75 Å and two shells of N or O at ~1.78 and ~1.92 Å. These distances are similar to those we have previously reported for native PSII preparations [Yachandra, V. K., Guiles, R. D., McDermott, A. E., Cole, J. L., Britt, R. D., Dexheimer, S. L., Sauer, K., & Klein, M. P. (1987) *Biochemistry* (following paper in this issue)] and are indicative of an oxo-bridged Mn complex. Our results demonstrate that the structure of the Mn complex is largely unaffected by removal of 33-, 24-, and 16-kDa extrinsic proteins, and thus these proteins do not provide ligands to Mn. The Mn K-edge spectrum of the $CaCl_2$-washed sample containing 2 Mn/PSII has a dramatically altered shape, and the edge inflection point is shifted to lower energy. The position of the edge is consistent with a Mn oxidation state of +3. The Mn EXAFS of this preparation is also quite different and cannot be simulated by using the parameters for the native Mn complex. Thus the structure of the Mn complex is disrupted upon depletion of half of the Mn.

Photosystem II (PSII)[1] catalyzes the light-driven oxidation of water to O_2 and the reduction of plastoquinone to plastoquinol. To accomplish the four-electron oxidation of water with one-electron photochemistry, PSII is thought to cycle through five successive intermediate states, S_i ($i = 0, ..., 4$), the S_4 state spontaneously decaying to produce S_0 and O_2 (Kok et al., 1970). A Mn-containing complex participates in the storage of oxidative equivalents and is thought to be the catalytic site for water oxidation [for reviews see Amesz (1983), Dismukes (1986), and Babcock (1987)]. The structure and oxidation states of this Mn complex in the various S states have been the subject of intense interest; EPR and X-ray absorption spectroscopies have proven to be particularly fruitful approaches.

PSII exists within the membrane as a complex of several hydrophobic intrinsic proteins and several hydrophilic extrinsic proteins. Despite considerable progress in our understanding of the structure of PSII, a clear picture of the Mn binding site has not yet emerged. It is generally assumed that the Mn complex is directly ligated to one or more PSII proteins. Although it is known from EXAFS studies that Mn is bound to terminal N, O ligands (Kirby et al., 1981a; Yachandra et al., 1987), the nature of the terminal ligands and the identity of proteins that constitute the binding site are not known. There are several papers claiming the isolation of a Mn-containing protein involved in O_2 evolution (Okada & Asada, 1983; Abramowicz & Dismukes, 1984). Biochemical studies of a non-O_2-evolving mutant of *Scenedesmus* suggest that the D1 protein, a 34-kilodalton (kDa) intrinsic protein associated with the reducing side of PSII, plays some role in Mn binding (Metz & Bishop, 1980; Metz et al., 1986).

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[1] Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; MES, 4-morpholineethanesulfonic acid; 2 Mn/PSII, $CaCl_2$-washed PSII sample containing two Mn per reaction center; 4 Mn/PSII, $CaCl_2$-washed PSII sample containing four Mn per reaction center; PSII, photosystem II; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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Several extrinsic membrane proteins are associated with O_2-evolution activity and thus may be involved in Mn binding [for reviews see Ghanotakis and Yocum (1985) and Murata and Miyao (1985)]. Proteins of 16 and 24 kDa are removed from PSII preparations by NaCl washing, resulting in partial inhibition of O_2 evolution (Åkerlund et al., 1982; Miyao & Murata, 1983; Ghanotakis et al., 1984a). However, all four Mn atoms remained bound to the membrane (Kuwabara & Murata, 1983; Ghanotakis et al., 1984a). In the absence of the 16- and 24-kDa proteins, high rates of O_2 evolution are observed with ≥30 mM Cl^- (Miyao & Murata, 1985) and bound Ca^{2+} (Miyao & Murata, 1984a; Ghanotakis et al., 1984b). Thus, these extrinsic proteins are associated with the binding of the cofactors Cl^- and Ca^{2+} but are not likely to be directly involved in Mn ligation.

PSII also contains a 33-kDa extrinsic protein (Kuwabara & Murata, 1982a) that may be released from PSII preparations or whole thylakoids by a number of procedures (Ghanotakis & Yocum, 1985), some of which release Mn and inactivate O_2 evolution. The relation of this protein to the Mn binding site remains controversial. It has been asserted that extraction of the 33-kDa protein under oxidizing conditions results in some binding of Mn to the isolated protein (Abramowicz & Dismukes, 1984; Yamamoto et al., 1984). However, extraction with 1 M $CaCl_2$ (Ono & Inoue, 1983) or 2.6 M urea and 200 mM NaCl (Miyao & Murata, 1984b) removes the 33-, 24-, and 16-kDa proteins and inhibits O_2 evolution, but does not release any Mn from the membrane. Interestingly, when assayed in the presence of 100–200 mM Cl^- and sufficient Ca^{2+}, preparations that retain all four Mn but are stripped of the three extrinsic proteins can evolve O_2 (Ono & Inoue, 1984; Miyao & Murata, 1984b), but the rate of the O_2 release reaction is greatly decreased (Miyao et al., 1987). Also, under these conditions the S_2 multiline EPR signal can be formed upon illumination (Miller et al., 1987; Styring et al., 1987). Thus, the 33-kDa protein facilitates turnover of the O_2-evolving complex, but is not absolutely required. Incubation at low Cl^- concentration results in the slow release of two Mn atoms from preparations lacking the 33-kDa protein (Ono & Inoue, 1984; Miyao & Murata, 1984b). These data indicate that the 33-kDa protein also functions to stabilize the binding of two Mn to a site on the membrane.

In this paper we examine the structural interactions between the 33-, 24-, and 16-kDa extrinsic proteins and the Mn complex using X-ray absorption spectroscopy. The shape and position of the X-ray absorption edge are sensitive to the oxidation state, types of ligands, and site symmetry in a coordination complex (Srivastava & Nigam, 1972). EXAFS probes the distance, identity, and number of atoms in the coordination environment of the absorbing atom [for reviews see Powers (1982) and Teo (1986)]. The Mn K-edge in PSII preparations exhibits a small preedge feature, which we assign to a 1s → 3d transition (Goodin et al., 1984; Yachandra et al., 1986), and for samples poised in the S_1 state by dark adaptation the edge inflection energy occurs at about 6551.3 eV, which is in the range observed for Mn^{3+} complexes. The results of our previous EXAFS studies on the Mn complex are consistent with an oxo-bridged binuclear structure, with a Mn–Mn distance of ~2.7 Å and N or O ligands at ~1.75 and ~2.0 Å (Kirby et al., 1981a; Goodin et al., 1984; Yachandra et al., 1986). We have collected Mn K-edge and EXAFS spectra of $CaCl_2$-washed PSII samples, which are depleted of the 33-, 24-, and 16-kDa extrinsic proteins and contain either four or two Mn per reaction center. We discuss the effects of removal of these extrinsic proteins on the structure of the Mn complex and directly address the relation of the 33-kDa extrinsic protein to the Mn binding site. We also discuss changes in the structure of the complex induced by release of two of the Mn atoms.

EXPERIMENTAL PROCEDURES

Sample Preparation. O_2-evolving PSII membranes were isolated from spinach by a modification of the Triton X-100 extraction procedures of Berthold et al. (1981) and Kuwabara and Murata (1982b) as previously described (Blough & Sauer, 1984). $CaCl_2$ washing was performed by incubating PSII samples at a Chl concentration of 1 mg/mL in 0.8 M $CaCl_2$ and 50 mM MES, pH 6.0 at 0 °C, for 2 h under ambient light. The membranes were recovered by centrifugation at 34000*g* for 10 min. To equilibrate the samples to low-salt concentration, the membranes were resuspended in 15 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES, pH 6.0, and centrifuged as above. The membranes were resuspended in the same buffer and centrifuged at 40000*g* for 1 h. Glycerol was added to the pellets to a concentration of about 30% (v/v), and samples were loaded into lucite holders, dark-adapted for 30 min, and stored at 77 K. The final Chl concentration was about 20–30 mg/mL. Note that turnover of the oxygen-evolving complex was blocked in these $CaCl_2$-washed samples because of the low (25 mM) Cl^- concentration in the medium (Ono & Inoue, 1983; Blough & Sauer, 1984; Cole & Sauer, 1987). To produce samples containing 2 Mn/PSII, $CaCl_2$-washed membranes, equilibrated in the low-salt buffer as above, were incubated at 4 °C for 18 h at a Chl concentration of 1 mg/mL. The membranes were washed twice in the same buffer to remove loosely bound Mn^{2+}, and X-ray absorption samples were prepared as described above.

Sample Characterization. Polyacrylamide gel electrophoresis was performed in the presence of 6 M urea and 0.1% SDS by the procedure of Laemmli (1970) as modified by Yamagishi and Katoh (1984). Heat treatment to release the 33-kDa protein was accomplished by incubating PSII membranes at a Chl concentration of 1.0 mg/mL in a buffer containing 50 mM MES (pH 6.0) at 55 °C for 5 min, followed by centrifugation at 15000*g* for 10 min. This procedure was performed in the absence of Cl^- because Nash et al. (1985) have reported that Cl^- depletion enhanced heat release of the 33-kDa protein. EPR spectra were taken directly in the X-ray sample holders at 8 K with a Varian E-109 spectrometer equipped with an Air Products Model LTR liquid helium cryostat. The EPR spectra were recorded both before and after X-ray exposure to check for release of Mn^{2+} or radiation damage. Mn content was assayed by flame atomic absorption spectroscopy. All of these analytical procedures were performed on the same samples used for X-ray spectroscopy.

X-ray Absorption Spectroscopy. Manganese X-ray absorption K-edge and EXAFS spectra were collected at the Stanford Synchrotron Radiation Laboratory, Stanford, CA, on beam lines IV-1 and VI-2 with Si[111] or Si[400] double crystal monochromators, respectively, during dedicated operation of the SPEAR storage ring. The sample temperature was maintained at 150–190 K during exposure to X-rays in a nitrogen gas flow cryostat. X-ray absorption spectra were recorded in a fluorescence mode (Jaklevic et al., 1977), using a plastic scintillator array similar to that described by Powers et al. (1981), equipped with a Cr filter and a Soller slit assembly to select for Mn fluorescence (Stern & Heald, 1979). Energy calibration was provided by simultaneous measurement of the "white line" preedge feature of $KMnO_4$ at 6543.3 eV (Goodin et al., 1979). The relative uncertainty in our edge energy measurements is ±0.1 eV.

The data were analyzed as described in detail elsewhere (Kirby et al., 1981a; Goodin, 1983; Yachandra et al., 1987), and only a brief summary will be presented here. A linear background was subtracted from the edge spectra, and edge inflection energies were determined by fitting the spectra to quadratic polynomials over 2.5–5.0-eV regions about each point and taking the first derivative of the smoothed spectra. The EXAFS modulation of the absorption, $\chi(k)$, is a function of the magnitude of the outgoing photoelectron wave vector k, which is given by

$$k = [2m_e(E - E_0)]^{1/2}/\hbar$$

where m_e is the electron mass, E is the incident X-ray photon energy, and E_0 is the edge energy. $\chi(k)$ was obtained from the raw data by subtracting a cubic spline fit to remove the isolated atomic and low-frequency background contribution and dividing by the free atom absorption. The background-subtracted data were weighted by k^1 and truncated at $k = 10$ or 10.8 Å^{-1}, and the data were then Fourier transformed. A window function was applied to isolate the R'-space Fourier peaks of interest prior to back-transformation to k space. This Fourier filtering method is used to remove the residual low-frequency background and high-frequency noise, thereby improving the precision of the fits. The back-transformed data were fit by the method of Teo and Lee (1979), which uses theoretical values for the amplitude and phase functions. The values of the average distance R, the number of scattering atoms N at distance R, the Debye–Waller factor σ, and the threshold energy E_0 were simultaneously fit with a nonlinear least-squares fitting program.

RESULTS

Sample Characterization. The $CaCl_2$-washed PSII samples were analyzed for residual amounts of the 33-, 24-, and 16-kDa proteins. It is difficult to determine the amount of the extrinsic 33-kDa protein in PSII membranes because there are several weakly staining intrinsic proteins in the 30-kDa region. Thus, we have also assayed the supernatant following heat treatment for residual 33-kDa protein. Figure 1 shows a Coomassie-stained SDS–urea gel of $CaCl_2$-washed and control PSII samples. The 33-, 24-, and 16-kDa proteins are not detectable in the $CaCl_2$-washed membranes, even when the lanes are overloaded (lanes 3 and 4). The weakly staining bands remaining in the 30-kDa region are also present in Tris-washed PSII preparations (data not shown). Heat treatment of control PSII samples released about half of the 33-kDa protein (compare lanes 5 and 7), as was previously reported by Nash et al. (1985). The proportion of 33-kDa protein released from the control preparation was not increased when it was heated to temperatures higher than 55 °C or when it was heated for longer than 5 min (data not shown). No 33-kDa band is detected in the pellet (lane 6) or supernatant (lanes 9 and 10) following heating of the $CaCl_2$-washed membranes. We estimate from these results that ≤5% of the 33-kDa protein remains bound to the $CaCl_2$-washed membranes.

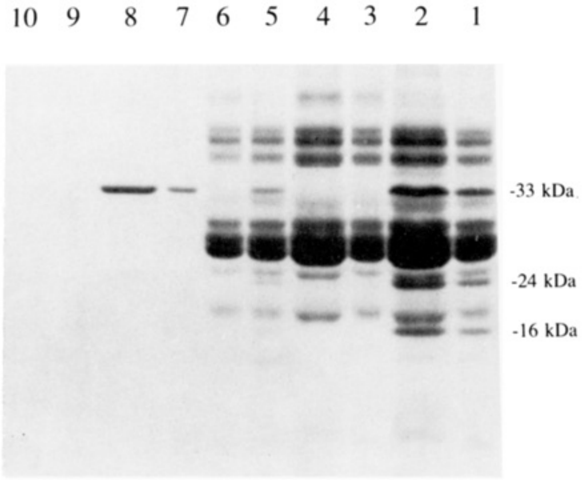

FIGURE 1: Polyacrylamide gel electrophoretogram of $CaCl_2$-washed PSII preparations. The samples were treated with 2.5% SDS, 8 M urea, and 5% mercaptoethanol for 1 h at room temperature and run on a separating gel containing 14% acrylamide, 0.1% SDS, and 6 M urea for 18 h. The gel was stained with Coomassie brilliant blue R250. The control (intact) PSII samples were not subjected to $CaCl_2$ washing. The lanes are (1, 2) control membranes, (3, 4) $CaCl_2$-washed membranes; (5) control, heat-treated membranes, (6) $CaCl_2$-washed, heat-treated membranes, (7, 8) supernatant of heat-treated control membranes, and (9, 10) supernatant of heat-treated $CaCl_2$-washed membranes. PSII membranes containing 10 (lanes, 1, 3, 5, 6) or 25 μg of Chl (lanes 2, 4) were loaded into the wells. Heat-treated PSII supernatants equivalent to 10 (lanes 7, 9) or 35 μg of Chl (lanes 8, 10) were loaded.

We also analyzed the $CaCl_2$-washed PSII membranes for Mn content. The antenna size of our PSII preparation is 260 ± 10 Chl; the integrated area of EPR signal II_{slow} was used to quantitate reaction centers (data not shown). The $CaCl_2$-washed PSII membranes contained 4.8 ± 0.4 Mn/260 Chl, as measured by atomic absorption spectroscopy. The control preparations not subjected to $CaCl_2$ washing also contained 4.8 ± 0.5 Mn/260 Chl. Thus, extraction of the extrinsic proteins was accomplished without removal of Mn. In contrast, incubation of $CaCl_2$-washed membranes in low ionic strength buffer for 18 h at 4 °C resulted in loss of Mn. Among several of these preparations the Mn content varied from 1.8 to 3.0 Mn/260 Chl. The samples within this group containing the higher total Mn content exhibited a large-amplitude six-line EPR signal characteristic of Mn^{2+}, whereas the EPR signal from the preparation containing 1.8 Mn/260 Chl accounted for a small proportion of the Mn (see below). This limiting value of 1.8 Mn/260 Chl is in good agreement with the results of Ono and Inoue (1984) and Miyao and Murata (1984b), who report that about 2 Mn/PSII remain bound after incubation at low ionic strength. Thus, the samples with the higher Mn content most likely contained adventitiously bound Mn^{2+}, and they were not used for X-ray spectroscopy.

The low-temperature EPR spectra of the dark-adapted $CaCl_2$-washed preparations containing either 4 or 2 Mn/PSII reaction center are shown in Figure 2. The large feature at $g = 4.3$ is due to high-spin rhombic Fe^{3+} and is commonly observed in PSII preparations. The turning points at about $g = 2.9$ and $g = 2.2$ arise from oxidized cytochrome *b*-559 that has been converted to the low-potential form by $CaCl_2$ washing, as previously observed by Matsuura and Itoh (1985). Both spectra contain a six-line signal due to Mn^{2+}, and the amplitude is greater in the 2 Mn/PSII sample. No other EPR signals that could be assigned to Mn^{2+} were detected in the 2 Mn/PSII samples over a temperature range of 4–25K (data not shown). We have calibrated the amplitude of the six-line Mn^{2+} signal under nonsaturating conditions (5 mW, room temperature) using a standard solution of $MnSO_4$. In the 2 Mn/PSII sample, the EPR-visible Mn^{2+} represents only 12–16% of the total Mn content; consequently, the bulk of the Mn is not detected with X-band EPR.

Mn K-Edge Spectra. The Mn X-ray absorption K-edge spectrum of a dark-adapted (S_1) control PSII sample, which retains the three extrinsic proteins, and the spectra of dark-adapted $CaCl_2$-washed samples, which contain 4 or 2 Mn/PSII, are displayed in Figure 3. The overall shape and

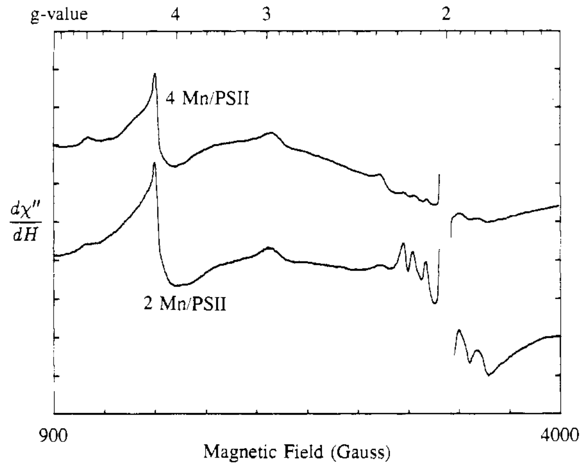

FIGURE 2: EPR spectra of $CaCl_2$-washed PSII samples. The region near $g = 2$ contains a large free radical signal and has been deleted. Instrument conditions: temperature, 8 K; microwave power, 50 mW; microwave frequency, 9.21 GHz; modulation amplitude, 32 G; modulation frequency, 100 kHz; scan time, 4 min; time constant, 128 ms.

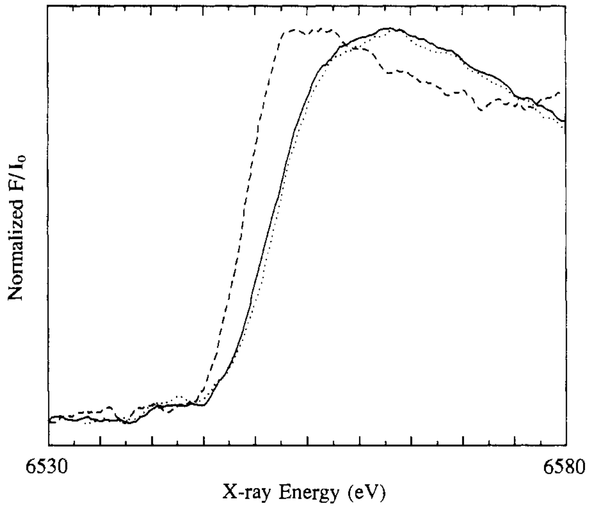

FIGURE 3: Mn K-edge spectra of $CaCl_2$-washed PSII preparations: control (intact) PSII (—) and $CaCl_2$-washed PSII containing 4 Mn/PSII (···) or 2 Mn/PSII (- - -). The spectra have been smoothed for presentation by linear regression over 1.4-eV regions about each point. The edge inflection energies are as follows: control, 6551.3 eV; 4 Mn/PSII, 6551.8 eV; 2 Mn/PSII, 6548.9 eV.

position of the edge of the 4 Mn/PSII sample are very similar to the control, and both spectra show a pronounced preedge transition at ~6543 eV. The K-edge inflection energy is 6551.8 eV for the 4 Mn/PSII sample and is 6551.3 eV for the control. In contrast, the spectrum of the 2 Mn/PSII sample is dramatically altered: the edge inflection energy is lowered to 6548.9 eV and the shape is changed. The signal-to-noise ratio is decreased in the 2 Mn/PSII spectrum due to the lower Mn concentration; thus, the preedge region is not resolved.

There are several possible explanations for the large decrease of the edge inflection energy of the 2 Mn/PSII spectrum relative to the 4 Mn/PSII and control spectra: (1) Mn^{2+} complexes have edge inflection energies of ~6547–6548 eV; thus, the presence of contaminating Mn^{2+}(aq) could shift the resulting composite edge to lower energy. (2) The oxidation state of the EPR-silent Mn is decreased to +2. (3) The oxidation state of the Mn is unchanged, but a ligand substitution has occurred, resulting in a less electronegative coordination environment. We have addressed the first possibility by simulating the composite edges of mixtures of Mn^{2+}(aq) and control (S_1) PSII. The edge created by adding 12% Mn^{2+} and 88% control PSII has an inflection energy of 6550.2 eV. Thus, the 12–16% Mn^{2+}(aq) detected by EPR is insufficient to shift the 2 Mn/PSII edge to 6548.9 eV. In fact, it is necessary to increase the Mn^{2+} content in the simulated edge to about 35% to obtain this inflection energy. We address possibilities 2 and 3 under Discussion.

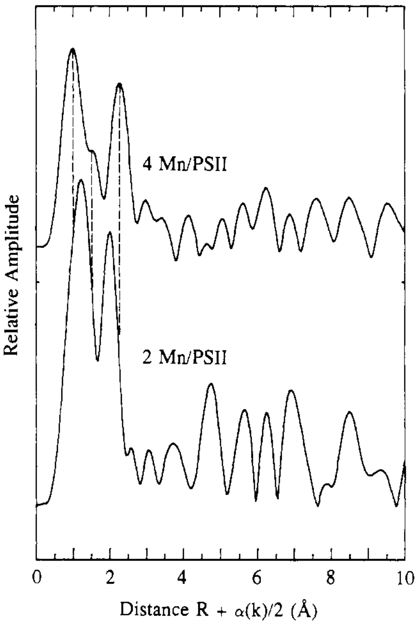

FIGURE 4: Fourier transforms of the k^1-weighted Mn EXAFS (3.0–10.0 or 10.8 Å^{-1}) of $CaCl_2$-washed PSII preparations. The FT peak positions are as follows: 4 Mn/PSII, 1.0, 1.5, and 2.2 Å; 2 Mn/PSII, 1.2 and 2.0 Å. The features at $R' > 4$ Å are due to noise. The fits to the 4 Mn/PSII data were performed according to the method of Teo and Lee (1979) and are described in the text and Table I.

Mn EXAFS Spectra. The FT spectra of the k^1-weighted Mn EXAFS of the 4 Mn/PSII and 2 Mn/PSII samples are displayed in Figure 4. In the 4 Mn/PSII spectrum, peaks at ~1.0, 1.5, and 2.2 Å are apparent, with the middle peak appearing as a shoulder on the first. Note that the peak position in the EXAFS FT spectra represents *apparent* distance R' and is shorter than the actual distance R because of the effect of the average phase shift $\alpha/2$. We have previously observed this pattern of three main peaks in the FT spectra of intact PSII preparations (Goodin et al., 1984; Yachandra et al., 1986, 1987). These three peaks were fit to bridging N, O ligands, terminal N, O ligands, and Mn, respectively.

The third peak is well resolved from the other two, and we have isolated and back-transformed this peak and have fit the resulting Fourier-filtered k-space EXAFS to theoretical phase and amplitude parameters. The best fit is obtained with Mn at a distance of 2.75 ± 0.03 Å, which is close to the Mn–Mn distance in intact PSII preparations (see Table I). The phases and amplitudes fit poorly to N, O or S, Cl scatterers (note that EXAFS cannot distinguish between elements that differ in atomic number by only one or two). EXAFS simulation also allows estimation of the number of ligands; however, the signal-to-noise ratio of the spectra of the $CaCl_2$-washed samples is fairly low, and we cannot obtain reliable values for ligand number from these data.

We have also isolated and back-transformed the three peaks together and have performed simultaneous three-shell fits to the Fourier-filtered 4 Mn/PSII EXAFS. The peaks at shorter distances are particularly sensitive to low-frequency background features, and thus the quality of these fits is lower. The two peaks at lower R' fit to N, O ligands at 1.78 ± 0.06 and 1.92 ± 0.07 Å, and the peak at higher R' fits to Mn at about

Table I: Comparison of Distances Extracted from the Simulation of Mn EXAFS of $CaCl_2$-Washed (4 Mn/PSII) or of Intact PSII Preparations[a]

	distance R (Å)[b]	
	$CaCl_2$ washed[c]	intact[d]
Mn–O or –N bridging ligand	1.78 ± 0.06	1.75 ± 0.05
Mn–O or –N-terminal ligand	1.92 ± 0.07	2.00 ± 0.05
Mn–Mn	2.75 ± 0.03	2.70 ± 0.03

[a] The parameters were obtained by the method of Teo and Lee (1979) as described under Experimental Procedures. The Debye–Waller factor σ used in the simulations was fixed at 0.022 Å, and E_0, which was initially chosen as the energy at the top of the edge, was allowed to vary ±20 eV. The coordination number of atoms in each shell, N, was within the range previously reported for intact PSII preparations (Yachandra et al., 1987). [b] The error limits are defined as the range of distances over which the least-squares residuals increase twofold over the minimum, with all other parameters in a given shell fixed. [c] This work. [d] Yachandra et al. (1987).

the same distance as was found in the fit to the isolated Mn shell. Consistent with these assignments, the amplitude of the third peak relative to the two peaks at lower R' increases on changing the weighting of the FT spectra from k^1 to k^2, indicating that the third peak arises from a higher Z scatterer, such as Mn (Kirby, 1981). These results are compared to those observed in intact PSII preparations in Table I. Although the bridging ligand distances are almost the same, the terminal ligand shell is about 0.1 Å shorter in the $CaCl_2$-washed sample. However, given the rather large uncertainty associated with this parameter, this difference may not be significant. Including a shell at ~2.4–2.5 Å containing one to four Cl^- atoms per four Mn in the simulation did not significantly improve the quality of the fit.

The FT spectrum of the 2 Mn/PSII sample is quite different from the 4 Mn/PSII or intact PSII spectra. Only two peaks at apparent distances of about 1.2 and 2.0 Å are present; these positions do not correspond to any of the three peaks in the 4 Mn/PSII spectrum. The Fourier-filtered EXAFS spectrum does not fit well to a combination of N, O ligands near 1.7 and 2.0 Å and Mn near 2.7 Å. Both Fourier peaks appear to contain contributions from several scatterers and possibly from background features. In particular, the second peak can be fit to several combinations of O, Cl, and Mn at distances of ~2.3, 2.4, and 2.6 Å, respectively, but it does not fit well to a Mn neighbor at 2.7 Å. It is possible that the two Mn atoms reside in different ligand environments, and the resultant EXAFS is a superposition of two different structures. Given this complexity and the low signal-to-noise ratio, we have not found a unique, physically reasonable fit to the 2 Mn/PSII EXAFS.

DISCUSSION

4 Mn/PSII. The results presented here demonstrate that the local structure of the Mn complex of PSII in the S_1 state is largely unaffected by the removal of the 33-, 24-, and 16-kDa extrinsic polypeptides. The presence of a neighboring Mn at ~2.7 Å and N, O ligands at ~1.7 and 2.0 Å is characteristic of the oxo-bridged multimeric Mn structure, which we have previously reported in intact PSII in the S_1 state (Yachandra et al., 1986, 1987). Also, the similarity of the shape of the Mn edge spectra from $CaCl_2$-washed and intact PSII samples is consistent with the preservation of the ligand environment of the Mn complex. The significance of the 0.5-eV higher edge inflection energy in the spectrum of the $CaCl_2$-washed sample is uncertain. It is possible that a terminal N is replaced by a more electronegative O ligand in the $CaCl_2$-washed sample, but otherwise the structure is intact. For a monomeric Mn complex such a substitution is expected to shift the edge ~0.4 eV to higher energy (Kirby et al., 1981b; Guiles et al., unpublished observations).

We conclude from our results that the amino acid side chains that presumably form the Mn binding site are not provided by the 33-kDa protein but by an intrinsic PSII protein(s). However, it is possible that the 33-kDa extrinsic protein supplies terminal N, O ligands to Mn that are replaced by others from an intrinsic membrane protein without appreciably changing the structure of the Mn complex, perhaps at the same time giving rise to the 0.5-eV shift in the edge energy described above. Even if this were the case, this model would imply a very nonspecific structural role for the 33-kDa protein.

The S_2 multiline EPR signal emanating from the Mn complex is sensitive to changes in the structure of the complex, and several groups have investigated the properties of this signal in PSII membranes lacking the extrinsic proteins (in the presence of sufficient Cl^- and Ca^{2+}). Hunziker et al. (1987) reported a decrease in the number of hyperfine lines in the multiline signal from PSII membranes lacking the 24- and 16-kDa proteins, and Styring et al. (1987) reported changes in the structure of the signal in preparations depleted of the 33-, 24-, and 16-kDa proteins. In contrast, Miller et al. (1987) did not observe changes in the EPR properties of the multiline signal upon removal of the 33-, 24-, and 16-kDa proteins. The results presented here indicate that the salient features of the structure of the Mn complex in the dark-adapted S_1 state are preserved in the absence of the extrinsic proteins, and we have previously shown that the $S_1 \rightarrow S_2$ transition in intact PSII does not involve an EXAFS-detectable change in the structure of the complex (Yachandra et al., 1987). Thus, from our EXAFS results it is likely that the structure of the Mn complex in the S_2 state is also largely insensitive to removal of the 33-, 24-, and 16-kDa proteins. However, it is possible that a small change in the structure of the Mn complex in the S_2 state could alter the properties of the multiline EPR signal without appreciably changing the Mn EXAFS.

In the present study the $CaCl_2$-washed PSII membranes were suspended at a low (25 mM) Cl^- concentration; under these conditions, turnover of the oxygen-evolving complex is blocked (Ono & Inoue, 1983; Blough & Sauer, 1984; Cole & Sauer, 1987). Thus, the mechanism by which the 33-kDa protein or high Cl^- levels facilitate secondary electron transfer on the oxidizing side of PSII does not involve any major changes in the structure of the Mn complex in the S_1 state.

2 Mn/PSII. The dramatically altered Mn K-edge and EXAFS spectra from the 2 Mn/PSII preparation indicate that the local environment of the remaining Mn atoms is quite different from that of the native Mn complex. Detailed comparison of the Mn coordination structure with the native Mn complex will require further EXAFS studies. It will be particularly interesting to determine whether the two Mn form a bridged binuclear complex; if not, they may still be close enough together to be detected by EXAFS (within about 4 Å). The structure of this site may provide insight into the structure of the native complex.

Despite the substantially lower edge inflection energy, the average oxidation state of Mn in the 2 Mn/PSII is most likely +3 for the following reasons: (1) The edge inflection energies of Mn^{2+} complexes range from ~6546.8 to 6548.0 eV (Kirby et al., 1981b), well below the 2 Mn/PSII edge, but the edge energy of Mn^{3+} superoxide dismutase (Goodin, 1983) is within 0.1 eV of the 2 Mn/PSII edge. The Mn ligands in this system are N and O, and it is possible that the Mn in the 2 Mn/PSII sample also exhibits a mixed ligand coordination environment. (2) The bulk of the Mn in the 2 Mn/PSII sample is EPR silent. Although Mn^{2+} bound to a protein in a low-symmetry ligand environment may give rise to a broad, anisotropic EPR spectrum (Reed & Markham, 1984), we have not detected any such EPR signals that could be assigned to Mn^{2+}. (3) Incubation of NaCl- or $CaCl_2$-washed PSII membranes with lipophilic reductants removes most of the Mn (Ghanotakis et al., 1984c; Tamura & Cheniae, 1986). Complexes of Mn^{3+} are much more stable than those of Mn^{2+} (Martell & Smith, 1974–1982), and the rate of ligand exchange for Mn^{3+} is much less than for Mn^{2+} (Margerum et al., 1978). Thus, in these PSII preparations Mn release probably occurs via reduction to the +2 oxidation state.

It has consistently been observed that only two of the four Mn remain bound to PSII membranes depleted of the 33-kDa protein and incubated at low chloride concentration (Ono & Inoue, 1984; Miyao & Murata, 1984b; vide supra). This heterogeneity in the pool of four Mn associated with PSII may arise via two possible mechanisms: (1) the environment of two of the Mn renders them less labile, either because the terminal ligands form stronger bonds to them, because they are stabilized by stronger bridging bonds, or because they are less accessible to the aqueous phase, or (2) two of the Mn are in a more stable oxidation state. We have proposed two different combinations of oxidation states for the four Mn in the S_1 state: (+3, +3, +3, +3) or (+3, +3, +4, +4) (Yachandra et al., 1987). In general, Mn^{4+} complexes are more reactive than Mn^{3+} complexes, owing to their higher redox potentials, and often decompose on exposure to water (Matsushita & Shono, 1983). Thus, assuming the second set of Mn oxidation states, the two Mn^{3+} ions remaining in the 2 Mn/PSII samples may correspond to the two Mn^{3+} ions present in native PSII, while the Mn released from the membrane may correspond to the two Mn^{4+} ions rendered labile upon removal of the extrinsic proteins.

The changes in the Mn complex that accompany depletion of the extrinsic proteins and two Mn atoms can be interpreted within the context of the two possible structures that we proposed on the basis of EXAFS studies of the native Mn complex: (1) a pair of equivalent binuclear complexes separated from one another by more than 3 Å or (2) a single binuclear complex with two other Mn atoms incorporated as monomers at distances greater than 3 Å (Yachandra et al., 1987). If structure 1 is correct, removal of two Mn that presumably formed one of the binuclear sites must alter the coordination environment of the remaining Mn. Alternatively, the labile Mn represents the monomer units of model 2, and the binuclear structure of the remaining two Mn is altered from the native complex. Another possibility is that the binuclear Mn of model 2 are extracted, leaving the two monomeric Mn behind. If the two monomer sites were different, the resulting EXAFS spectrum could be quite complex. In conclusion, the results presented here are compatible with models 1 or 2, and in either case it is possible that structural coupling between the four Mn atoms results in a structural rearrangement upon removal of half of the Mn.

From our results we conclude the following: (1) The structure of the Mn complex of PSII is essentially unchanged upon depletion of the 33-, 24-, and 16-kDa extrinsic proteins. (2) The 33-kDa extrinsic protein is not directly involved in binding the Mn complex of PSII. (3) The Mn coordination environment is altered in PSII preparations that contain only two Mn.

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