Structure and Orientation of the Mn$_4$Ca Cluster in Plant Photosystem II Membranes Studied by Polarized Range-extended X-ray Absorption Spectroscopy*§**

Yulia Pushkar*†, Junko Yano*†‡, Pieter Glatzel*, Johannes Messinger**‡, Azul Lewis‡§, Kenneth Sauer‡§, Uwe Bergmann**, and Vittal Yachandra†‡

From the †Melvin Calvin Laboratory, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720-5230, the ‡Department of Chemistry, University of California, Berkeley, California 94720-5230, the §European Synchrotron Radiation Facility, Grenoble Cedex 38043, France, the **Stanford Synchrotron Radiation Laboratory, Menlo Park, California 94025, and the ***Max-Planck-Institut für Bioanorganische Chemie, D-45470 Mülheim an der Ruhr, Germany

X-ray absorption spectroscopy has provided important insights into the structure and function of the Mn$_4$Ca cluster in the oxygen-evolving complex of Photosystem II (PS II). The range of manganese extended x-ray absorption fine structure data collected from PS II until now has been, however, limited by the presence of iron in PS II. Using a crystal spectrometer with high energy resolution to detect solely the manganese K-edge fluorescence, we are able to extend the extended x-ray absorption fine structure range beyond the onset of the iron absorption edge. This results in improvements in resolution of the manganese-backscatterer distances in PS II from 0.14 to 0.09 Å. The high resolution data obtained from oriented spinach PS II membranes in the S$_2$ state show that there are three di-$\mu$-oxo-bridged manganese-manganese distances of $\sim$2.7 and $\sim$2.8 Å in a 2:1 ratio and that these three manganese-manganese vectors are aligned at an average orientation of $\sim$60° relative to the membrane normal. Furthermore, we are able to observe the separation of the Fourier peaks corresponding to the $\sim$3.2 Å manganese-manganese and the $\sim$3.4 Å manganese-calcium interactions in oriented PS II samples and determine their orientation relative to the membrane normal. The average of the manganese-calcium vectors at $\sim$3.4 Å is aligned along the membrane normal, while the $\sim$3.2 Å manganese-manganese vector is oriented near the membrane plane. A comparison of this structural information with the proposed Mn$_4$Ca cluster models based on spectroscopic and diffraction data provides input for refining and selecting among these models.

Photosynthesis by green plants, algae, and cyanobacteria provides essentially all of the dioxygen in the biosphere as a byproduct of the electron transfer processes utilizing water as the ultimate electron source: 2H$_2$O $\rightarrow$ O$_2$ + 4H$^+$ + 4e$^-$. Water oxidation is a light-driven reaction that is catalyzed by an oxygen-evolving complex (OEC)$^4$ of Photosystem II (PS II) (1–4). The active site of the OEC is known to be a protein-bound complex containing four manganese and one calcium atom. This complex cycles through a series of five intermediate redox states that are referred to as S states (S$_0$ to S$_5$) (5). The S state transitions are driven by successive light-induced one-electron oxidations of the PS II reaction center. In each step the complex accumulates oxidizing equivalents until dioxygen is released during the spontaneous return from S$_5$ to S$_0$.

Many of the proposed mechanisms of water oxidation depend critically on knowledge of the Mn$_4$Ca cluster structure. To date, structural models of the OEC complex have been suggested based on EPR techniques (6–9), x-ray absorption spectroscopy (XAS) (10–14), x-ray diffraction (XRD) (15–17), and infrared spectroscopy (Fourier transform infrared) (18). The XRD studies (3.0–3.8 Å resolution) have located the Mn$_4$Ca cluster in the density map (16, 17) and confirmed the presence of calcium in the OEC cluster, as had been shown previously by EPR (19–21) and by extended x-ray absorption fine structure (EXAFS) spectroscopy (22, 23). A recent XAS study showed that the OEC complex is very susceptible to reduction and disruption during x-ray exposure, under the conditions used in collecting the published XRD data (24). Consequently, the precise location of the manganese and calcium atoms has not been reliably established within active OEC centers by XRD, as acknowledged in the most recent study (17).

Manganese XAS enables a detailed analysis of the Mn$_4$Ca cluster in the OEC. X-ray absorption near-edge structure (XANES) contains information on the electronic structure and changes in oxidation states of the manganese that accompany S state transitions (25). EXAFS allows for a precise determination

---

* This work was supported in part by National Institutes of Health Grant GM-55302 and by the Director, Office of Science, Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences and of the Department of Energy under Contract DE-AC02-05CH11231. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This article was selected as a Paper of the Week.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental data (including Equations S1–S7), Figs. S1–S8, Tables S1–S3, and Refs. 1–9.

1 To whom correspondence may be addressed: 1 Cyclotron Rd., Calvin Laboratory, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720. Tel.: 510-486-4330; Fax: 510-486-6059; E-mail: jjano@lbl.gov.

2 Supported by the Deutsche Forschungsgemeinschaft (Me 1629/2-3).

3 To whom correspondence may be addressed: 1 Cyclotron Rd., Calvin Laboratory, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720. Tel.: 510-486-4330; Fax: 510-486-6059; E-mail: vkychandra@lbl.gov.

4 The abbreviations used are: OEC, oxygen-evolving complex; EXAFS, extended x-ray absorption fine structure; FT, Fourier transform; PS II, photosystem II; XANES, x-ray absorption near edge spectroscopy; XAS, x-ray absorption spectroscopy; XRD, x-ray diffraction; MES, 4-morpholineethanesulfonic acid.
Structure and Orientation of the Mn$_4$Ca Cluster in Photosystem II

![Diagram](Image 60x567 to 396x734)

FIGURE 1. **Range-extended x-ray absorption spectroscopy.** Left, x-ray fluorescence of manganese and iron; Above, manganese K$_{α1}$ and K$_{α2}$ fluorescence peaks, with natural line width of $\sim$5 eV, split by 11 eV. The multilayer monochromator with 1-eV resolution is tuned to the manganese K$_{α1}$ peak. Below, fluorescence peaks of manganese and iron as detected using germanium detector. The fluorescence peaks are convoluted with the electronic window resolution of 150–200 eV of the germanium detector. This method of detection cannot resolve manganese K$_{α1}$ and K$_{α2}$ fluorescence peaks. Note different energy scales for the schemes shown above and below. Iron is an obligatory element in functional PS II complexes. (Below) Comparison of the PS II manganese K-edge EXAFS spectrum from an S$_1$ state PS II sample obtained with a traditional 30-element crystals from cyanobacteria, using an x-ray dose below the threshold of damage, has derived feasible structures for the Mn$_4$Ca cluster and the orientation of the cluster in the PS II membrane. The strontium-reactivated PS II membranes was used to predict the orientations of the individual manganese-manganese vectors (superposition of mono-oxo-bridged manganese-manganese and manganese-calcium vectors) have been reported in two studies (34, 35), whereas another study reported an average angle of $80 \pm 10^\circ$ for the $\sim$2.7 Å vectors without providing results for the $\sim$3.3 Å vector (36). Because of the limited resolution, conventional EXAFS is not able to determine the orientations of the individual manganese-manganese and manganese-calcium vectors in the 3.2–3.4 Å region. In a complementary study, strontium K-edge polarized EXAFS of strontium-reactivated PS II membranes was used to predict the manganese-calcium orientation. It showed a lower and upper limit of 0 and $23^\circ$, respectively, for the average angle between the manganese-strontium vector(s) and the membrane normal and yielded an isotropic coordination number of manganese neighbors to strontium of either one or two (23). A recent polarized x-ray absorption spectroscopy study of PS II single crystals from cyanobacteria, using an x-ray dose below the threshold of damage, has derived feasible structures for the Mn$_4$Ca cluster and the orientation of the cluster in the PS II crystal (14).

In this work, we applied range-extended EXAFS to study the dichroism of the Mn$_4$Ca cluster in oriented PS II membranes from spinach chloroplasts. The study shows: (i) the separation of the manganese-manganese ($\sim$3.2 Å) and manganese-calcium ($\sim$3.4 Å) vectors, which allows independent analysis of...
their orientation relative to the membrane normal; (ii) the determination of the dichroism characteristics of the three short manganese-manganese vectors (two at 2.7 Å and one at 2.8 Å) and their orientation in the PS II membrane. These results are used to discuss the structure and orientation of the Mn$_4$Ca cluster in the PS II membrane.

MATERIALS AND METHODS

Sample Preparation and Characterization—PS II samples were prepared from spinach as previously described (37). They typically contain 4 manganese per 200–250 chlorophylls. The oxygen evolution rates for the PS II samples used in this study are between 400 and 500 μmol O$_2$/(mg chlorophylls · h). The membranes were resuspended in 50 mM MES buffer, pH = 6.0, containing 0.4 M sucrose and 5 mM CaCl$_2$ and pelleted by centrifugation at 4°C (39,000 × g, 1 h). One or two drops of 50 mM MES buffer were added to the pellet, and the resulting paste was painted onto Mylar tape. The PS II membranes were dried under a stream of cold nitrogen gas at 4°C in the dark for ~1 h, as described previously (38). This process was repeated five to seven times to generate samples with a sufficiently thick sample layer for the x-ray absorption experiment.

The paint-and-dry cycles produce one-dimensionally ordered samples with a preferred orientation of the PS II membrane normal perpendicular to the substrate surface. The extent of orientation (mosaic spread, which is the half-width of the Gaussian distribution of the angle of the membrane normal to the substrate normal in the PS II samples) was assessed from the angle dependence of the Tyr Dox and cytochrome b$_{559}$ EPR signals (see supplemental data). X-band EPR spectroscopy was performed with a Varian E-109 spectrometer, a standard TE$_{102}$ cavity, and an Air Products liquid helium cryostat. The samples used in this study displayed a mosaic spread of 15–20°. After drying the samples, their integrity was assayed by monitoring the amount of S$_2$ multilane signal formed upon sample illumination at 195 K. The amplitude of the manganese signal was the same as that obtained from randomly oriented membranes at a state (germanium) detector was placed at the common focus of the four crystals on the intersecting Rowland circles. The analyzer energy was tuned to the manganese Kα peak at 5899 eV at a Bragg angle of 74.84°. A nitrogen-cooled solid state (germanium) detector was placed at the common focus of the four crystals on the intersecting Rowland circles. The analyzer bandwidth of 0.8 eV was determined by measuring the elastically scattered peak.

Experimental procedures and limitations for measuring range-extended EXAFS past multiple K- or L-edges, and the design and operation of the spectrometer have been described previously (32, 39). All samples were measured below 10 K in a liquid He cooled cryostat (Oxford CF1208).

Data Analysis—For each EXAFS scan, the energy was calibrated using the KMnO$_4$ pre-edge reference peak (6543.3 eV), and the intensity was normalized by I$_0$, before averaging. Approximately 1000 scans were averaged for each orientation of PS II membranes relative to the x-ray e-vector with a custom Matlab program. Data reduction of the EXAFS spectra was done as described previously (10, 40). Curve fitting was performed using ab initio calculated phases and amplitudes from the FEFF8 program from the University of Washington (41). These phases and amplitudes were used in the EXAFS Equation 1, which is described below and contains a sinusoidal function that gives the distance and an amplitude function that contains information about the scattering atom and the number of such neighboring atoms.

$$
\chi(k) = S_0^2 \sum_j \frac{N_j}{k R_j^2} f_{\text{ab}}(\pi, k, R_j) e^{-2a^2 k^2} e^{-2R_j a/k} \times \sin(2k R_j + \alpha(k)) \quad \text{(Eq. 1)}
$$

The neighboring atoms to the central atom(s) are divided into j shells, with all atoms with the same atomic number and distance from the central atom grouped into a single shell. Within each shell, the coordination number $N_j$ denotes the number of neighboring atoms in shell j at a distance of $R_j$ from the central atom, $f_{\text{ab}}$ is the ab initio amplitude function for shell j, and the Debye-Waller term $e^{-2a^2 k^2}$ accounts for damping due to both static and thermal disorder in absorber-back-
Structure and Orientation of the Mn\textsubscript{4}Ca Cluster in Photosystem II

scatterer-distances. The mean free path term $e^{-2R_j/\lambda(k)}$ reflects losses due to inelastic scattering, where $\lambda(k)$ is the electron mean free path. The oscillations in the EXAFS spectrum are reflected in the sinusoidal term $\sin(2kR_{ij} + \alpha_j(k))$, where $\alpha_j(k)$ is the \textit{ab initio} phase function for shell $j$. This sinusoidal term shows the direct relation between the frequency of the EXAFS oscillations in $k$-space and the absorber-backscatterer distance. The EXAFS equation (Equation 1) was used to fit the experimental Fourier isolates using $N$, $R$, and $\sigma^2$ as variable parameters. Fit details and evaluation of fit qualities are given in the supplemental data.

The spatial resolution in EXAFS is inversely related to the spectral range. Several formulas can be found in the EXAFS literature describing the resolution limits of the method, such as $\Delta R \Delta k \approx 1$, $\Delta R_{\text{max}} = \pi/2$ and $\Delta R \Delta k = \pi/2$ (40, 42); for more details see the supplemental data (40).

For a detailed explanation of the theory of polarized EXAFS see the supplemental data. Angle $\theta$ is the angle between the x-ray $e$-vector and the membrane normal, and $\phi$ denotes the relative orientation of the manganese-backscatterer (manganese-manganese or manganese-calcium) vector of interest to the membrane normal.

RESULTS

Manganese X-ray Absorption Spectra—X-ray absorption manganese K-edge spectra of oriented membranes in the $S_1$ state are shown in Fig. 2. Data were collected for two orientations in which the sample normal is placed at either 15 or 75° to the direction of the $e$-vector of the polarized x-rays. The edge positions and post-edge shape exhibit a marked angle dependence, as reported previously for oriented PS II membranes (34). Dichroism of the manganese K-edge spectra is even more obvious in the second-derivative spectra (Fig. 2, bottom). The powder manganese XANES spectrum created from the spectra collected from oriented membranes at two different orientations is identical to that obtained from a frozen solution sample. This result provides independent confirmation that the oriented samples are intact and not damaged by x-rays.

The $k^3$-weighted EXAFS spectra of the $S_1$ state of PS II oriented with the membrane normal at 15 or 75° to the x-ray $e$-vector are shown in Fig. 3. The spectra are distinctly dichroic. The region of the photoelectron wavevector from 3.5 to 11.5 Å\textsuperscript{-1} (denoted by the dashed line in Fig. 3), which is accessible by conventional EXAFS, agrees with results published earlier for oriented PS II membranes in the $S_1$ state (34). Clear differences can be seen in the spectra between the two orientations.

Fig. 4A shows the Fourier transforms of the range-extended EXAFS ($k^3$-weighted) at 15 or 75°. The Fourier transforms exhibit well defined peaks, labeled I, II, IIIA, and IIIB, corresponding to the shells of backscatterers at different “apparent” distances, $R'_i$, from the manganese absorber. The apparent distance is shorter than the actual distance due to a phase shift induced by the interaction of the given absorber-scatterer pair with the photoelectron. For comparison, the Fourier transforms of the range-extended EXAFS spectra of both orientations, but truncated at 11.5 Å\textsuperscript{-1}, are shown in Fig. 4B. Significant improvement in spectral resolution is observed for the range-extended EXAFS data (Fig. 4A). Increased spectral resolution reveals the orientation dependence of peaks II and IIIA and IIIB. The intensity of peak II, which consists of three manganese-manganese distances at 2.7 and 2.8 Å (see below) changes significantly between 15 and 75°, with higher intensity at 75°. Peak III shows a complex nature containing at least two peaks, IIIA and IIIB, with distances of 3.2 and 3.4 Å, respec-
Structure and Orientation of the Mn₄Ca Cluster in Photosystem II

The Fourier transforms shown in Fig. 4, A and C, provide the basis for drawing qualitative conclusions about possible distances in the manganese-backscatterer pairs and their preferential orientations relative to the membrane normal. Reliable quantitative results can be obtained by fitting the experimental data using the EXAFS Equation 1, as described under “Materials and Methods” and in the supplemental data. The assignment of each peak and a detailed analysis of the actual distance and orientation of each vector are described below. We will concentrate on FT peaks II and III, because these are from manganese-manganese and manganese-calcium backscattering and provide the most reliable information about the Mn₄Ca structure and orientation.

Curve Fitting of EXAFS FT Peak II—Fits of Fourier peak II were carried out both separately and in conjunction with peak III (peaks II + III). The large differences in amplitude and envelope shapes of the oscillation of the peak II (and III) isolates at the two different orientations reflect the dichroism observed in the Fourier transform amplitudes (Fig. 5). The quality of the fits is judged using the fit error parameters Φ and e²; to know how they are determined see the supplemental data (note that e² is normalized to the number of fit parameters). Fit error parameters reflect the deviation between the simulations and isolates. Fits 1–4 in Table 1 show the results from fitting one and two manganese-manganese shells to peak II at the 15° and 75° orientations of the S₁ state. Addition of the second manganese-manganese shell (fits 3 and 4 in Table 1) results in considerable improvement of the fit quality. With two different manganese-manganese distances at 2.7 and 2.8 Å, the fit error Φ and e² decreased by ~40% for the two-shell fit relative to the one-shell fit. In our previous range-extended EXAFS study with isotropic solution samples we concluded that peak II is best interpreted as consisting of 2.7 Å and 2.8 Å manganese-manganese vectors (di-μ-oxo-bridged manganese-manganese moieties) with a 2:1 ratio in both S₁ and S₂ states (27). Our present results on oriented samples support this conclusion.

The N_app values are higher for the 75° orientation compared with those for the 15° orientation. The orientation dependence of data extracted with a one-shell fit of Peak II (Tables 1 and
supplemental Table S1, fits 1 and 2) results in $N_{\text{iso}} = 1.3 \pm 0.3$, which corresponds to two or three manganese-manganese interactions at an average angle of $\langle \phi \rangle = 63 \pm 5^\circ$, for a 2.74 ± 0.02 Å manganese-manganese vector. Note that two manganese-manganese vectors correspond to $N_{\text{iso}} = 1.0$, which is at the lower border of the error bar; taking into account that the EXAFS technique tends to underestimate $N$ values, the $N_{\text{iso}} = 1.3 \pm 0.3$ obtained favors three manganese-manganese interactions (expected $N_{\text{iso}} = 1.5$). The angle is in agreement with that reported earlier based on conventional polarized EXAFS data (34, 35). The new range-extended data show that peak II contains interactions at 2.7 and 2.8 Å, which can be analyzed separately (Table 1, fits 3 and 4). Fig. 6 shows linear plots of $N_{\text{app}}$ derived from fits 3 and 4 in Table 1 (solid squares) against $3\cos^2\theta - 1$ (see supplemental Equation S6). Third points (open squares) were obtained from extended EXAFS measurements of isotropic PS II $S_1$ in solution (see supplemental Table S1). Linear fits using only two data points from oriented samples (solid lines) or using three data points including the isotropic values (dashed lines) are nearly identical and result in the same $N_{\text{iso}}$ and $\langle \phi \rangle$ values. Supplemental Fig. S7 shows more traditional polar plots of the $N_{\text{app}}$ derived from fits 1 to 4 in Table 1 and supplemental Table S1 and plotted with respect to the detection angle ($\theta$). Analysis of the orientation dependence of the 2.72 ± 0.02 Å manganese-manganese vector results in $N_{\text{iso}} = 0.88 \pm 0.2$ (two manganese-manganese interactions) at an average angle $\langle \phi \rangle = 61 \pm 5^\circ$, with respect to the membrane normal. The 2.83 ± 0.02 Å manganese-manganese vector exhibits $N_{\text{iso}} = 0.46 \pm 0.12$ (one manganese-manganese interaction) at an angle $\langle \phi \rangle = 64 \pm 10^\circ$ with respect to the membrane normal.

**Curve Fitting of EXAFS FT Peak III**—Curve fitting results for peak III are shown in Table 2. Analysis of peak III has been problematic in EXAFS studies of PS II because of the relatively weak intensity and correspondingly low signal-to-noise ratio. As mentioned above, peak III was found to exhibit dichroism and to consist of at least two different manganese-backscatterer vectors (34, 35). Both of these conclusions are strengthened by the new range-extended EXAFS measurements. Combinations of manganese, calcium, carbon, and oxygen were tested to fit peak III (10, 34, 40). Distances of 3.1–3.4 Å are reported between manganese atoms bridged by a single $\mu_3$- or $\mu_3$-oxo unit. In addition, for carboxylate- or histidine-derived ligands, a highly disordered shell of carbon atoms at 2.9–3.3 Å may be expected from the next-nearest neighbor atoms to the metal (46–51). However, attempts to include manganese-light atom (oxygen or carbon) shells into peak III fits result in increased fit errors (data not shown). On the basis of differences in apparent distances ($R'$), dichroism for peak IIIA and IIIB, and previous studies of oriented strontium-reactivated PS II membranes (23), we conclude that the manganese-calcium vector contributes mainly at 15° and the manganese-manganese vector at 75°. One-shell fits presented in Table 2 (rows 1 and 2) agree well

**TABLE 1**

One- and two-shell fits of Fourier Peak II of oriented PS II membranes ($S_1$ state) at angles of 15° and 75° between the membrane normal and the e-vector of the linearly polarized x-ray beam

| Fit # | Angle  | Shell              | $R$(Å) | $N_{\text{app}}$ | $a^2(\text{Å}^2) \times 10^6$ | $\Phi \times 10^5$ | $\epsilon^2(\times 10^6)$ |
|-------|--------|--------------------|--------|------------------|------------------------------|-------------------|------------------------|
| One shell | 1      | 15° Manganese-Manganese | 2.73    | 0.94             | 3.0                          | 0.20              | 0.068                  |
|        | 2      | 75° Manganese-Manganese | 2.74    | 1.48             | 3.6                          | 0.18              | 0.063                  |
| Two shells | 3      | 15° Manganese-Manganese | 2.72    | 0.70             | 1b                           | 0.12              | 0.040                  |
|        | 4      | 75° Manganese-Manganese | 2.82    | 0.33             | 1b                           | 0.11              | 0.036                  |
|        |       | Manganese-Manganese   | 2.72    | 0.97             | 1b                           |                   |                        |
|        |       | Manganese-Manganese   | 2.83    | 0.54             | 1b                           |                   |                        |

*Errors are estimated to be 25% for $N_{\text{app}}$ numbers.
*Parameter was fixed.
with the above assignment and support a longer distance for the manganese-calcium vector compared with that for the manganese-manganese vector. Estimation of the minor contributions of the manganese-calcium vector at 75° orientation and the manganese-manganese vector at 15° orientation is required to determine the orientations of those vectors relative to the membrane normal, but isolating peaks of weak intensities results in unreliable two-shell fits. As described in an earlier study (23), we can either assume an $N_{\text{app}} = 0$ for contributions close to the noise level or estimate the upper limit of the $N_{\text{app}}$ based on proportionality of the reduced amplitude of FT peak IIIA or IIIB to the peak maximum at the same $R'$ in the complementary orientation. When the measured amplitude was close to the calculated noise level (between 4 and 10 Å in the FTs), as for the manganese-calcium-interaction, the noise level in the FT spectrum was used to estimate the upper limit of $N_{\text{app}}$. The upper limits of the $N_{\text{app}}$ for manganese-manganese and manganese-calcium interactions are listed in Table 2.

Fig. 7 shows $N_{\text{app}}$ for the manganese-manganese component of Peak III derived from Table 2 plotted against $3\cos^2\theta - 1$, as was done for Peak II in Fig. 6. A third point ($open squares$) was obtained from extended EXAFS measurements of isotropic PS II $S_1$ in solution (supplemental Table S2). A linear fit using only two data points from oriented samples ($solid lines$) and a fit using three data points including the isotropic values ($dashed lines$) are similar and, within experimental error, result in similar $N_{\text{iso}}$ and $\langle \phi \rangle$ values. The orientation dependence of $N_{\text{app}}$ for the manganese-calcium $3.2 \pm 0.02$ Å vector (see Table 2) results in $N_{\text{iso}} = 0.39 \pm 0.1$ and $\langle \phi \rangle = 70°$. These values are consistent with a single manganese-calcium $3.2$ Å vector, for which the expected value of $N_{\text{iso}}$ is 0.5.

For the manganese-calcium $3.4$ Å distance, the expected value for $N_{\text{iso}}$ is 0.25 for one manganese-calcium vector or 0.50 for two vectors. If we assume that $N_{\text{app}} = 0$ at 75°, the angle dependence of $N_{\text{app}}$, for this component results in $N_{\text{iso}} = 0.29 \pm 0.09$ for $\langle \phi \rangle = 0°$. This value favors one manganese-calcium interaction at $3.40 \pm 0.02$ Å; however, taking into account the error range and previous data of Cinco et al. (22) the possibility of two interactions at this distance cannot be excluded (22). The upper limit of this angle was estimated to be 18° in this work and 23° by Cinco et al. (23) for the strontium-manganese interaction. Despite relatively high uncertainty, we can conclude that this vector is aligned near to the membrane normal.

**FIGURE 6.** Linear plot of polarized manganese EXAFS data from Fourier peak II. Linear plots of the x-ray absorption dichroism of Peak II for oriented PS II samples in the S1 state. The $N_{\text{app}}$ values ($solid squares$) are derived from two-shell curve fits of FT peak II (Fig. 4A and Table 1) and are plotted against $3\cos^2\theta - 1$ (see supplemental Equation S6). Best fits are shown for the different manganese-manganese vectors as $solid lines$ and a fit using three data points including the isotropic values ($dashed lines$) are very close to those obtained using only oriented sample data.

**TABLE 2**

Fits of Fourier Peak III of oriented PS II membranes ($S_1$ state) at angles of 15 and 75° between the membrane normal and the e-vector of the linearly polarized x-ray beam

| Fit # | Angle | Shell          | $R$(Å) | $N_{\text{app}}$ | $\alpha^2$ $(Å^2) \times 10^4$ | $\Phi$ $(\times 10^3)$ | $\epsilon^2$ $(\times 10^4)$ |
|-------|-------|----------------|--------|-----------------|-------------------------------|------------------------|--------------------------|
| Peak III isolate |       |                |        |                 |                               |                        |                          |
| 1     | 15°   | Manganese-calcium | 3.37   | 0.77$^a$       | $2^b$                         | 0.42                   | 0.14                     |
| 2     | 75°   | Manganese-manganese | 3.24   | 0.46$^a$       | $2^b$                         | 0.49                   | 0.17                     |
|       | 15°   | Manganese-manganese | 3.24   | 0.22$^a$       |                               |                        |                          |
|       | 75°   | Manganese-calcium  | 3.11$^d$|                 |                               |                        |                          |

$^a$ Errors are estimated to be 25% for $N_{\text{app}}$ numbers.

$^b$ Parameter was fixed.

$^c$ $N_{\text{app}}$ was estimated from relative peak heights in the Fourier transform.

$^d$ The $N_{\text{app}}$ was estimated from noise level in the Fourier transform.
interactions. For the native \( S_1 \) and \( S_2 \) states, with less distance heterogeneity, the results of the conventional EXAFS were still not conclusive (40).

The recent range-extended EXAFS study of PS II in solution provides evidence supporting the presence of three di-\( \mu \)-oxo-bridged manganese-manganese vectors in the native \( S_1 \) and \( S_2 \) states (27). The conclusion is based on the fits of the Fourier peak II and II + III isolates, which demonstrated that: (i) two distinct manganese-manganese vectors contribute to peak II (2.7 and 2.8 Å); (ii) there is an unequal distribution of the coordination numbers of \( N_1 \) (2.7 Å) and \( N_2 \) (2.8 Å), which would be consistent with the presence of three di-\( \mu \)-oxo-bridged manganese-manganese moieties; (iii) the fit clearly improved when the \( N_1/N_2 \) ratio is close to 2:1 with \( N_{\text{tot}} \approx 1.5 \). The difference between the two di-\( \mu \)-oxo-bridged manganese-manganese distances is approximately 0.1 Å for both the \( S_1 \) and the \( S_2 \) state, which explains why the traditional EXAFS study with a distance resolution of 0.14 Å was unable to reveal such distance heterogeneity.

The current polarized EXAFS data from oriented PS II membranes in the \( S_1 \) state support the conclusions summarized above for the \( S_1 \) state in solution. The fit qualities for peak II shown in Table 1 demonstrate significant improvement if two manganese-manganese vectors are introduced. The x-ray absorption linear dichroism from oriented PS II membranes demonstrates that the average manganese-manganese (\( \sim 2.7 \) Å) vector and manganese-manganese (\( \sim 2.8 \) Å) vector both have similar orientation of \( \sim 60^\circ \) to the membrane normal, as summarized in Table 3. The averaged (\( \sim 2.7–2.8 \) Å) vector is oriented at \( \sim 63^\circ \), which is the same as the value reported earlier from conventional EXAFS studies with oriented PS II membranes (34).

Several possible reasons for manganese-manganese distance heterogeneity can be suggested: 1) differences in the redox states of manganese atoms resulting in the 2.7 Å and 2.8 Å manganese-manganese vectors; 2) differences in types of \( \mu \)-oxo bridges (\( \mu_2 \)-oxo versus \( \mu_3 \)-oxo) connecting manganese atoms; 3) protonation of the \( \mu \)-oxo bridge. Protonation of the \( \mu \)-oxo bridge lowers the manganese-oxygen bond order, which causes an increase in the manganese-manganese distance. Studies of model compounds support these possible reasons for distance heterogeneity (59 – 61).

FT Peak III; Orientation of the Long Manganese-Manganese and Manganese-Calcium Vectors Relative to the Membrane Normal—Fits to EXAFS data allow consideration of some relevant questions about the chemical nature of backscatterers contributing to peak III and, now, about the orientation of those manganese-backscatterer vectors relative to the membrane normal. Calcium was included in the fit combination because it has been implicated as a structural element of the OEC through \( O_2 \) evolution activity (19, 45, 62–66), EPR (20), and calcium- and strontium-EXAFS experiments (22, 23). In conventional EXAFS experiments it was noticed previously that the addition of the manganese-calcium vector to the manganese-manganese long interaction improves fit qualities. The evidence that peak III cannot be a result of only manganese-calcium interactions came from a study in which the manganese EXAFS spectrum of calcium-depleted PS II showed a peak III with decreased intensity (43). High resolution range-extended EXAFS data on oriented PS II membranes provide new experimental support for the conclusion that peak III contains both manganese-manganese and manganese-calcium interactions; the combination of oriented preparations and range-extended EXAFS allows the

![Figure 7. Linear plot of polarized manganese EXAFS data from Fourier peak III.](image)

**TABLE 3**

| Vector R(Å)         | \( N_{\text{app}} \) | Angle to membrane normal, (φ) |
|---------------------|----------------------|--------------------------------|
| Peak II, one shell fit | Manganese-manganese  | 2.74 ± 0.02                      | 1.30 ± 0.30  | 63 ± 5°   |
| Peak II, two shell fit | Manganese-manganese  | 2.74 ± 0.02                      | 0.88 ± 0.20  | 61 ± 5°   |
|                      | Manganese-calcium    | 3.30 ± 0.02                      | 0.39 ± 0.10  | >70°      |
| Peak III, estimation of the \( N_{\text{app}} \) limits are included | Manganese-manganese  | 3.40 ± 0.02                      | 0.29 ± 0.09  | <18° (<23)° |

* Value from Cinco et al. (23).
Structure and Orientation of the Mn$_4$Ca Cluster in Photosystem II

FIGURE 8. Cluster models compatible with polarized range-extended EXAFS. A, models for the Mn$_4$Ca cluster compatible with the range-extended manganese EXAFS data with three short 2.7–2.8 Å manganese-manganese distances and one longer manganese-manganese distance at 3.2 Å. B, Mn$_4$ models developed from Fig. 8A topological core structures and their proposed orientation relative to the membrane normal consistent with polarized range-extended EXAFS (Table 3). Note that in the membrane plane there is a rotational ambiguity which is always present for one-dimensionally oriented samples such as layered membranes. We emphasize that there may be other models that can be tested in this manner (this would include structural and optical isomers of listed models). C, the orientation of the average manganese-calcium vector in relation to the 3.2 Å manganese-manganese vector. The cones represent a range for the average manganese-calcium vector(s) along the membrane normal (−18°), and the 3.2 Å manganese-manganese vector toward the membrane plane (−20°), respectively.

two types of interactions to be resolved. Information about the relative orientation of the manganese-strontium vectors was reported in the earlier study of strontium-reconstituted, oriented PS II membranes (23).

Results of analysis of the x-ray absorption linear dichroism from peak III of the oriented PS II samples are summarized in Table 3. The orientation of the averaged manganese-calcium vector is similar to that reported for strontium-reconstituted, oriented PS II membranes (23). The manganese-manganese (3.2 Å) vector is oriented more toward the membrane plane. The average of both vectors is in agreement with the results from conventional EXAFS: 43° for the 3.3 Å vectors (combination of mono-μ-oxo-bridged manganese-manganese and manganese-calcium vectors) (34).

Structure and Orientation of the Mn$_4$Ca Cluster in PS II—The structural information about the Mn$_4$Ca cluster in the S$_1$ state, based on polarized EXAFS data, is summarized in Table 3. Topological models for the Mn$_4$ cluster compatible with the EXAFS data (27) and containing three short 2.7–2.8 Å manganese-manganese vectors and one manganese-manganese interaction at 3.2 Å are shown in Fig. 8A. Previously, dichroism characteristics of only the averages of the short manganese-manganese vectors at ~2.7 Å and the long manganese-manganese and manganese-calcium interactions at ~3.3 Å were known (34). Those data were not sufficient to restrict the possible orientations of the proposed models with respect to the PS II membrane or the PS II protein frame. With the new range-extended EXAFS data, we can for the first time determine independently the angle dependence for three different (2.8 Å, averaged 2.7 Å, and 3.2 Å) manganese-manganese vectors relative to the membrane normal. This allows us to impose additional restrictions on proposed structural models. The importance of the results in Table 3 is that they restrict the angles of the manganese-manganese or manganese-calcium vectors relative to the membrane normal for all three manganese-manganese vectors simultaneously.

Knowledge of the angles between the membrane normal and each of three different vectors involving manganese-manganese interactions allows us to determine whether one or more orientations for any particular model are consistent with the dichroism data. For this purpose we used the averaged 2.7, 2.8, and 3.2 Å manganese-manganese vectors, and we illustrate this approach for each of the models in Fig. 8A; the orientations shown in Fig. 8B are in agreement with the dichroism measurements. We emphasize that there may be other models that can be tested in this manner (this would include structural and optical isomers of listed models). There are two possibilities for the placement of the 2.8 Å manganese-manganese vector for Model I and three possibilities for Model II. As structures Ia and Ib and IIa, IIb, and IIc in Fig. 8B demonstrate, different placement of the 2.8 Å manganese-manganese vectors results in rather small changes in the orientation of models, as follows from the close values of the angle of 2.8 Å manganese-manganese vector and averaged angle of two 2.7 Å manganese-manganese vectors to the membrane normal (60°, Table 3). Uncertainty in the angle between the 3.2 Å manganese-manganese vector and membrane normal (>70°, Table 3) results in a subset of model orientations as this angle changes within the determined range; however, this does not produce dramatic changes in the model orientations. Model III has a high degree of rotational freedom for the 3.2 Å manganese-oxygen-manganese mono-μ-oxo unit that results in multiple solutions; in Fig. 8B we show only one such example. There are many possibilities for the placement of calcium in the models shown in Fig. 8B, and the average orientation of the manganese-calcium vector can best be described to be within a cone about the membrane normal, as shown in Fig. 8C. Using the results of polarized EXAFS (Table 3), the range of possible orientations of the models to the membrane normal can be dramatically reduced as illustrated in Fig. 8B. However, the following uncertainties remain: (i) rotational ambiguity in the membrane plane, which is always present for one-dimensionally oriented samples such as oriented membranes and (ii) multiple possibilities for calcium coordination; present data do not allow us to distinguish clearly whether there is one or two manganese-calcium interactions.
as well as precise angle between the manganese-calcium vector and the membrane normal.

The geometric information obtained from polarized EXAFS measurements on spinach membranes provides an important tool for testing proposed Mn₄Ca models from other studies. For example, results from this study can be compared with the models from X-ray crystal structures from cyanobacteria. The PS II x-ray structures at 3.5 and 3.0 Å resolution (16, 17) indicate three manganese-calcium interactions at 3.2–3.4 Å distances, with average manganese-calcium angles of ~40° and ~34° to the membrane normal for the two different structures, respectively (16, 17). Those angles are larger than the upper limit from this study of ~18° and from the strontium-reconstituted PS II membrane study (23) of ~23°. In Ferreira et al. (16) two manganese-manganese interactions at ~3.2 Å were modeled in the OEC structure with an average angle of ~48° to the membrane normal, which is different from the lower limit of ~70° from this study. In Loll et al. (17), the ~3.2 Å manganese-manganese interactions form an average angle of ~61°, which is closer to the results in this study. However, the OEC in Loll et al. (17) contain two ~2.7 Å and two ~3.2 Å manganese-manganese vectors, compared with the three ~2.7 Å and one ~3.2 Å manganese-manganese distances required by EXAFS data.

Recently the polarized EXAFS measurements on PS II single crystals of the thermophilic cyanobacterium Thermosynechococcus elongatus combined with XRD resulted in a set of high resolution structures for the Mn₄Ca cluster (14). Model I from Yano et al. (14) has all three (averaged 2.7, 2.8, and 3.2 Å) manganese-manganese vectors oriented differently relative to the membrane normal compared with those determined in this study (Table 3) and is less favored (14). Models II and III from Yano et al. (14) are similar to IIa in this study (Fig. 8B), with the ~60° orientation of the 2.8 Å manganese-manganese vector and ~80° orientation of the 3.2 Å manganese-manganese vector relative to the membrane normal; however, the averaged orientation of the 2.7 Å manganese-manganese vectors to the membrane normal is ~40° compared with ~60° (Table 3). This difference in orientation of the averaged 2.7 Å vectors with respect to the membrane normal could be due to the inherent errors in the determination of the angles in this method, or we speculate that this variation in orientation could reflect the differences in PS II from thermophilic cyanobacteria versus spinach. Also note that the resolution of the two experiments is different; analysis of the single crystal EXAFS data involve considerations of the protein unit cell symmetry, while oriented membranes have a unique axis along the membrane normal with rotational uncertainty in the plane of the membrane.

Range-extended EXAFS provides an important technical development that allows differentiation of the 2.7 and 2.8 Å manganese-manganese, the 3.2 Å manganese-manganese, and the 3.4 Å manganese-calcium interactions. Detailed information about the orientation of manganese-manganese and manganese-calcium vectors in the OEC in the Sₗ state provide a critical starting point for the analysis of the structural changes in the OEC throughout the catalytic S state cycle.

Acknowledgments—We thank Prof. Stephen P. Cramer at the Lawrence Berkeley National Laboratory for suggesting the range-extended EXAFS experiment and are grateful to him for providing access to the crystal analyzer (National Institutes of Health Grant GM-65440, National Science Foundation Grant CHE-0213592, and Office of Biological and Environmental Research, Department of Energy (to S. P. Cramer)). Dr. Olga Krupina is acknowledged for helpful discussions. Synchrotron facilities were provided by Advanced Photon Source operated by the Department of Energy, Office of Basic Energy Sciences under Contract W-31-109-ENG-38. BioCAT is a National Institutes of Health-supported Research Center (Grant RR-08630). Stanford Synchrotron Radiation Laboratory is operated by Stanford University for the United States Department of Energy, Office of Basic Energy Sciences.

REFERENCES

1. Debuss, R. J. (1992) Biochim. Biophys. Acta 1102, 269–352.
2. Rutherford, A. W., Zimmermann, J.-L., and Boussac, A. (1992) in The Photosystems: Structure, Function, and Molecular Biology (Barber, J., ed) pp. 179–229, Elsevier Science Publishers B.V., Amsterdam.
3. Ort, D. R., and Yocum, C. F. (eds) (1996) Oxidogenic Photosynthesis: The Light Reactions, Kluwer Academic Publishers, Dordrecht, The Netherlands.
4. Wydrzynski, T., and Satoh, S. (2005) Photosystem II: The Light-Driven Water-Plastoquinone Oxidoreductase, Springer, Dordrecht, The Netherlands.
5. Kok, B., Forbush, B., and McGloin, M. (1970) Photochem. Photobiol. 11, 457–475.
6. Miller, A. F., and Brudvig, G. W. (1991) Biochim. Biophys. Acta 1056, 1–18.
7. Hasegawa, K., Ono, T. a., Inoue, Y., and Kusunoki, M. (1999) Chem. Phys. Lett. 300, 9–19.
8. Carrell, T. G., Tyrshkin, A. M., and Dismukes, G. C. (2002) J. Biol. Inorg. Chem. 7, 2–22.
9. Brit, R. D., Campbell, K. A., Peloquin, J. M., Gilchrist, M. L., Aznar, C. P., Dicus, M. M., Robblee, J., and Messinger, J. (2004) Biochim. Biophys. Acta 1655, 158–171.
10. DeRose, V. J., Munkerji, L., Latimer, M. J., Yachandra, V. K., Sauer, K., and Klein, M. P. (1994) J. Am. Chem. Soc. 116, 5239–5249.
11. Yachandra, V. K., Sauer, K., and Klein, M. P. (1996) Chem. Rev. 96, 2927–2950.
12. Pfenninger, J. E. (1999) Struct. Bond. 90, 1–36.
13. Sauer, K., Yano, J., and Yachandra, V. K. (2005) Photosynth. Res. 85, 73–86.
14. Yano, J., Kern, J., Sauer, K., Latimer, M., Pushkar, Y., Biesiadka, J., Loll, B., Saenger, W., Messinger, J., Zouni, A., and Yachandra, V. K. (2006) Science 314, 821–825.
15. Kamiya, N., and Shen, J. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 98–103.
16. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) Science 303, 1831–1838.
17. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Nature 438, 1040–1044.
18. Chai, H.-A., Hillier, W., Law, N. A., and Babcock, G. T. (2001) Biochim. Biophys. Acta 1503, 69–82.
19. Boussac, A., and Rutherford, A. W. (1988) Chem. Sci. 28A, 123–126.
20. Boussac, A., and Rutherford, A. W. (1988) Biochemistry 27, 3476–3483.
21. Boussac, A., Zimmermann, J.-L., and Rutherford, A. W. (1989) Biochem. Biophys. Acta 9884–8989.
22. Cinco, R. M., Robblee, J. H., Yano, J., Pizarro, S. A., Bellacchio, E., Sauer, K., and Yachandra, V. K. (2002) Biochemistry 41, 12928–12933.
23. Cinco, R. M., Robblee, J. H., Messinger, J., Fernandez, C., Holman, K. L. M., Sauer, K., and Yachandra, V. K. (2004) Biochemistry 43, 13271–13282.
24. Yano, J., Kern, J., Irgang, K.-D., Latimer, M. J., Bergmann, U., Glätzel, P., Pushkar, Y., Biesiadka, J., Loll, B., Sauer, K., Messinger, J., Zouni, A., and Yachandra, V. K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 12047–12052.
Structure and Orientation of the Mn₄Ca Cluster in Photosystem II

25. Messinger, J., Robblee, J. H., Bergmann, U., Fernandez, C., Glatzel, P., Visser, H., Cinco, R. M., McFarlane, K. L., Bellacchio, E., Pizarro, S. A., Cramer, S. P., Sauer, K., Klein, M. P., and Yachandra, V. K. (2001) J. Am. Chem. Soc. 123, 7804–7820

26. Yachandra, V. K. (2005) in Photosystem II: The Light-Driven Water-Plastoquinone Oxidoreductase (Wydrzynski, T., and Satoh, S., eds) pp. 235–260, Springer, Dordrecht, The Netherlands

27. Yano, J., Pushkar, Y., Glatzel, P., Lewis, A., Sauer, K., Messinger, J., Bergmann, U., and Yachandra, V. K. (2005) J. Am. Chem. Soc. 127, 14974–14975

28. Scott, R. A. (1984) in Structural and Resonance Techniques in Biological Research (Rousseau, D., L., ed) pp. 295–362, Academic Press, Orlando, FL

29. Cramer, S. P. (1988) J. Phys. Chem. B 102, 8257–8265

30. Pizarro, S. A., Visser, H., Cinco, R. M., Robblee, J. H., Pal, S., Mukhopadhyay, S., Mok, H. J., Sauer, K., Wieghardt, K., Armstrong, W. H., and Yachandra, V. K. (2004) J. Bio. Inorg. Chem. 9, 247–255

45. Boussac, A., Rappaport, F., Carrier, P., Verbabatz, J. M., Gobin, R., Kirillovsky, D., Rutherford, A. W., and Sugiuira, M. (2004) J. Biol. Chem. 279, 22809–22819

56. Kusunoki, M., Takano, T., Ono, T., Noguchi, T., Yamaguchi, Y., Oyanagi, H., and Inoue, Y. (1995) in Photosynthesis: from Light to Biosphere (Mathis, P., ed) pp. 251–254, Kluwer Academic Publishers, Dordrecht, The Netherlands

63. Grove, G. N., and Brudvig, G. W. (1998) Biochim. Biophys. Acta 1351–1352

64. Ono, T.-A., and Inoue, Y. (1988) FEBS Lett. 231–234

65. Mukhopadhyay, S., Mandal, S. K., Bhaduri, S., and Armstrong, W. H. (2004) Chem. Rev. 104, 3981–4026

66. Ananyev, G. M., and Dismukes, G. C. (1997) Biochim. Biophys. Acta 1351–1352

7208 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 10 • MARCH 9, 2007