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Where Water Is Oxidized to Dioxygen: Structure of the Photosynthetic Mn₄Ca Cluster

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Abstract

The oxidation of water to dioxygen is catalyzed within photosystem II (PSII) by a Mn₄Ca cluster, the structure of which remains elusive. Polarized extended x-ray absorption fine structure (EXAFS) measurements on PSII single crystals constrain the Mn₄Ca cluster geometry to a set of three similar high-resolution structures. Combining polarized EXAFS and x-ray diffraction data, the cluster was placed within PSII, taking into account the overall trend of the electron density of the metal site and the putative ligands. The structure of the cluster from the present study is unlike either the 3.0 or 3.5 angstrom-resolution x-ray structures or other previously proposed models.

Oxygen, which makes up about 20% of Earth's atmosphere, comes mostly from photosynthesis that occurs in cyanobacteria, green algae, and higher plants (1). These organisms have within photosystem II (PSII) an oxygen-evolving complex (OEC), in which the energy of sunlight is used to oxidize water to molecular oxygen. The heart of the OEC is a cluster of four Mn atoms and one Ca atom (Mn₄Ca) connected by mono-μ-oxo, di-μ-oxo, and/or hydroxo bridges. The specific protein environment and one chloride ion are also essential for the water-splitting activity (1). During the oxidation of water, the OEC cycles through five different oxidation states, which are known as S_i states (where *i* ranges from 0 to 4), that couple the one-electron photochemistry of the PSII reaction center with the four-electron chemistry of water oxidation (2).

The structure of the Mn₄Ca cluster and its role in the mechanism of water oxidation have been investigated with the use of spectroscopic methods (1), especially electron

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paramagnetic resonance and electron nuclear double-resonance spectroscopy (3–9), x-ray spectroscopy (10), and Fourier transform infrared (FTIR) spectroscopy (11). In addition, recent x-ray diffraction (XRD) studies of single crystals of PSII provide critical information about its structure at 3.8 to 3.0 Å resolution (12–16). However, even XRD data of the highest resolution presently available are insufficient to accurately determine the positions of Mn, Ca, and the bridging and terminal ligands. This is reflected by the differences in the placement of the metal ions and putative ligands in the 3.0 (16) and 3.5 Å (14) structures. Furthermore, at the x-ray dose and temperature used in the XRD studies, the geometry of the Mn₄Ca cluster is disrupted, initiated by the rapid reduction of Mn(III) and Mn(IV) present in the dark-stable S₁ state to Mn(II), as shown by Mn x-ray absorption near-edge structure (XANES) studies and Mn x-ray absorption fine structure (EXAFS) studies of PSII single crystals (17).

EXAFS experiments with PSII require a substantially lower x-ray dose than XRD measurements (17), and the onset of radiation damage can be precisely determined and controlled by monitoring the Mn K-edge position, thus allowing the collection of data from the intact Mn₄Ca cluster of PSII. In addition, EXAFS provides metal-to-metal and metal-to-ligand distances with high accuracy (~0.02 Å) and a resolution of ~0.1 Å. Mn and Ca EXAFS studies of frozen solutions of PSII preparations have provided accurate distances and numbers of Mn-Mn, Mn-Ca, and Mn/Ca-ligand vectors in the Mn₄Ca cluster (10, 18–21) and have led to the development of several possible structural models for the Mn₄Ca cluster (fig. S1).

For polarized EXAFS experiments on one-dimensionally oriented membranes or three-dimensionally oriented single crystals, the EXAFS amplitude is orientation dependent and proportional to $\sim \cos^2\theta$, where θ is the angle between the **e**-field vector of the polarized x-ray beam and the absorber-backscatterer vector. Therefore, this technique provides important additional geometric information about the metal site in metalloprotein crystals (22, 23). Here we show that polarized EXAFS can be used to provide structural models of the Mn₄Ca active site, revealing details currently unresolvable by XRD. Combining information from polarized EXAFS and XRD (16) leads to the placement of these models within the PSII protein environment.

PSII crystals from *Thermosynechococcus elongatus* in the dark-stable S₁ state were oriented so that the x-ray **e**-field vector was parallel to the *a*, *b*, or *c* axis of the crystal unit cell. The total photon dose on the sample was kept at a safe level of 1×10^7 photons/μm² on the basis of detailed radiation damage studies on single crystals of PSII (17). After the Mn XANES and EXAFS spectra were collected, the orientation of the crystal was determined in situ by the collection of x-ray diffraction patterns (fig. S2) (24).

Figure 1A (top) shows the polarized Mn K-edge XANES spectra of PSII single crystals with the x-ray **e**-field vector parallel to each of the orthogonal crystal unit cell axes (*a*, *b*, and *c*). The spectra show distinctive features at each orientation, both in the main K edge (1s to 4p) and the pre-edge region [1s-to-3d transitions (Fig. 1A, inset)]. The orientation-dependent differences are more clearly seen in the second-derivative plots (Fig. 1A, bottom).

The Fourier transforms (FTs) of the polarized Mn EXAFS of PSII single crystals are shown in Fig. 1B. Peak I has contributions from bridging and terminal O or N atoms at 1.8 to 2.0 Å; peak II results from di-μ-oxo-bridged Mn-Mn interactions at 2.7 to 2.8 Å; and peak III contains contributions from mono-μ-oxo-bridged Mn-Mn and Mn-Ca interactions at 3.3 and 3.4 Å, respectively. The EXAFS spectra show a pronounced dependence on the crystal orientation, indicating that the Mn₄Ca cluster is highly asymmetric.

A test of whether the redox state and the structure of the Mn_4Ca cluster in the single crystals are equivalent to that in active oxygen-evolving PSII solution samples is to calculate the isotropic solution spectra (powder spectra) from the single-crystal spectra and compare these to experimental solution spectra. The comparison of XANES and EXAFS spectra in Fig. 2, A and B (with the corresponding second derivatives for XANES in Fig. 2A, bottom), shows that the spectra are indistinguishable within the extremely low noise of these experimental data. This result confirms the dichroism of the EXAFS spectra and shows that the cluster geometry is not disrupted by radiation damage. Therefore, the data of Fig. 1B form a reliable basis for the elucidation of the structure and orientation of the Mn_4Ca cluster within PSII.

Initially, we used the polarized EXAFS data to test the Mn_4Ca structures proposed on the basis of XRD data (14, 16). The comparison of the experimental data (Fig. 1B) with the polarized EXAFS spectra calculated for the XRD models at 3.5 (14) and 3.0 Å (16) resolution is shown in fig. S3. The disagreement is notable; it is most likely due to the limited resolution and x-ray damage to the Mn_4Ca cluster during XRD measurements (17).

In the next step, the polarized EXAFS data were used to evaluate and filter the large number of previously proposed and feasible models for the Mn_4Ca cluster [Mn_4 motifs **a** to **r** (fig. S1)], including topologically related structures [the motifs from the XRD structures are **c** (16) and **n** (14)]. For this filtering process, the polarized EXAFS for the proposed structural models was calculated as follows (fig. S4) (24): (i) Each model was placed into one PSII monomer, (ii) the coordinates for the companion monomer were determined by means of the local C_2 symmetry of the dimeric PSII complex, (iii) the coordinates for the four symmetry-related dimeric units within the $\text{P2}_1\text{2}_1\text{2}_1$ crystal unit cell were determined, and (iv) the EXAFS spectrum was calculated for the PSII single crystal with the **e** vector of the x-ray beam parallel to each of the crystal unit cell axes (*a*, *b*, and *c*) with the use of the FEFF8 program (25). To determine the optimal orientation, we rotated each of the inserted clusters in a stepwise fashion within the PSII monomer with respect to the three axes *x*, *y*, and *z* [for definition of axes, see Fig. 3 (top right)]. For each orientation, the EXAFS spectra were calculated and compared to the experimental results shown in Fig. 1B. This process was continued until the rotational space was mapped sufficiently to determine whether a specific model complied with the polarized EXAFS data (24). The focus in our comparison was predominantly on the contribution to the EXAFS from Fourier peaks II and III, which are from Mn-Mn and Mn-Ca interactions that mainly define the motif for the Mn_4Ca cluster. This process rules out unsatisfactory cluster geometries with a high degree of confidence.

Range-extended EXAFS results (21) show that three short Mn-Mn distances between 2.7 and 2.8 Å are present in the Mn_4Ca cluster. The presence of the 2.8 Å Mn-Mn vector is observed as a shift of FT peak II to a longer distance along the *a* axis in the polarized EXAFS data (Fig. 1B). In addition to the 2.8 Å vector, two 2.7 Å vectors are required to reproduce the intensity of the FT peak II, mainly along the *b* and *c* axes (see the EXAFS fit parameters in table S1). Therefore, the models containing only two short 2.7 Å Mn-Mn distances, or one 2.7 Å and one 2.8 Å Mn-Mn distances [motifs **a** to **j** (fig. S1A)], were unacceptable in reproducing both the solution range-extended EXAFS (21) and polarized EXAFS data.

The remaining models contain three Mn-Mn vectors at 2.7 to 2.8 Å and 0, 1, or 2 Mn-Mn vectors at 3.3 Å [motifs **k** to **r** (fig. S1B)]. The Mn EXAFS solution of isotropic frozen PSII solution samples shows that FT peak III is fit best by one Mn-Mn vector at 3.3 Å and two Mn-Ca vectors at 3.4 Å (10), which is supported by Sr and Ca EXAFS results (19, 20). Models with these distances [motifs **p** to **r** (fig. S1B)] and models containing two 3.3 Å Mn-Mn distances [motifs **l** to **o** (fig. S1B)] along with the Mn-Ca distances were included in our search for a match with the observed dichroism (a heterogeneity of ~0.2 Å was included for

models with two 3.3 Å Mn-Mn vectors to decrease the otherwise too-intense contribution to FT peak III).

The application of the rigorous testing protocol described above showed that the polarized EXAFS data (Fig. 1B) are consistent with three topologically related structures [models I, II, and III (Fig. 3)] that are based on the Mn₄ motif **r** (Fig. 3, top) (18, 21). These models are shown in the orientation (indicated by the axis system shown in Fig. 3, top right) required to satisfy the polarized EXAFS data. The experimental polarized spectra (Fig. 1B) and the calculated spectra from the three structures positioned as shown (Fig. 3, left panel) are very similar with regard to the intensity and the orientation dependence for FT peak II and III, which determine the motif of the structural model. The trend in the dichroism is also similar for FT peak I. Most of the contribution to peak I is from bridging O atoms, because Mn-terminal ligand distances are highly disordered (10, 18) (table S1) and hence were not included in the simulations. The ambiguities in the intensities in FT peak I, however, come from the small contributions from this disordered shell of terminal ligands, which are difficult to model.

At present, it is not possible to choose among the three models on the basis of the polarized EXAFS data. In addition, each of these structural models can have four symmetry-related orientations in the membrane originating from the $\cos^2\theta$ dependence of the EXAFS signal and the noncrystallographic C_2 symmetry of the monomers in the PSII dimer (24).

To further distinguish between these structural options, we used the following criteria: (i) the placement of the Ca atoms; (ii) the distribution of the electron density from XRD (16), which is pear-shaped, with the narrow end oriented toward the dimer C_2 axis; and (iii) the information from the position of putative amino acid ligands identified around the electron density coinciding with the Mn₄Ca cluster (16). The placement of Ca relied on Sr EXAFS data showing that the two Mn-Sr(Ca) vectors are along the membrane normal (20) and the anomalous XRD studies indicating that Ca is above the Mn plane toward the redox-active tyrosine Y_Z and P₆₈₀ chlorophylls (14, 16). The placement of Ca reduces the options among the symmetry-related orientations by half. The remaining two symmetry-equivalent orientations of models I, II, and III were examined for compliance with the overall trend of the electron density and the placement of ligands (24). The best orientations of the three models within the PSII membrane are shown in Fig. 3. The other symmetry-related orientation is clearly unsatisfactory because of the pronounced asymmetry in the electron density.

The best-fit ligand environment was obtained for all the models [coordinates in table S2 (24)], but there was some overlap between the cluster and ligand positions (fig. S6). The ligand assignments must remain tentative at this point because movement of the protein ligand residues can result from radiation damage to the metal site during XRD (17). In the XRD study, the Mn close to Asp¹⁷⁰ (i.e., Mn_A) and Ca have a lower occupancy (σ value) as compared to those of the other Mn atoms (16). This lower value might indicate that the Mn_A and Ca metal sites are more susceptible to radiation damage and hence their ligands may be prone to movement. The ligand environment for the best-fit orientation for model II is shown in Fig. 4.

The considerable differences in the orientation and structures between model II and those in the recent XRD proposals (14, 16) for the Mn₄Ca complex are shown as an overlay in fig. S7 (24). This highlights the difficulties of deriving high-resolution structures from low-resolution XRD. Although constraints from EXAFS distance information are included in the building of the XRD model that lead to approximate structures, there are too many structures that can be fit to the low-resolution electron density. The importance of polarized EXAFS of

single crystals lies in its ability to discriminate between the many possible high-resolution models by relying on the dichroism of the EXAFS spectra.

The structural changes of the Mn_4Ca complex on advancing through the S_i state intermediates can be placed in the context of the polarized EXAFS data to assist in deriving a mechanism for photosynthetic water oxidation. The FTIR data, in conjunction with model II (Fig. 4), suggest that Mn_A , which may be ligated by Asp^{170} , does not change oxidation state and remains Mn(III) or Mn(IV) throughout the Kok cycle. The C-terminal Ala^{344} may be a ligand to Mn_D , which is proposed to undergo $\text{Mn(III)} \rightarrow \text{Mn(IV)}$ oxidation during the $S_1 \rightarrow S_2$ transition (26–28). Recent FTIR data suggest that His^{332} monitors structural changes of the Mn_4Ca cluster, but no evidence for a Mn-centered oxidation was reported (29). Because Mn_C is closer to His^{332} , Mn_C may remain Mn(III) or Mn(IV) throughout the cycle. Consequently, Mn_B is a likely candidate for Mn oxidation during the $S_0 \rightarrow S_1$ transition.

The dichroism in the polarized EXAFS data from single crystals provides a powerful filter for choosing among many of the proposed structural models. Also, as shown in this study, the combination of XRD and polarized EXAFS on single crystals has several advantages for unraveling structures of x-ray damage-prone, redox-active metal sites in proteins. XRD structures at medium resolution are sufficient to determine the overall shape and placement of the metal site within the ligand sphere, and refinement by means of polarized EXAFS can provide accurate metal-to-metal and metal-to-ligand vectors. In addition, different intermediate states of the active site (including different metal oxidation states), which may be difficult to study with XRD at high resolution, can be examined. The structural model from polarized EXAFS from the S_1 state presented here, and from the other S states, will provide a reliable foundation for the investigation of the mechanism of photosynthetic water oxidation and for the design of biomimetic catalysts for water splitting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) Single-crystal, polarized Mn K-edge XANES spectra (top) and the corresponding second derivatives of the XANES spectra (bottom) of PSII in the S_1 state. The XANES spectra are shown with the x-ray $\vec{e}$ -field vector aligned parallel to the principal crystal axes from single crystals of PSII [a (red curve), b (blue curve), and c (green curve)] from *T. elongatus*. The inflection point of the edges and the shape of the spectra are clearly dependent on the orientation of the single crystal with respect to the x-ray $\vec{e}$ vector. The inset shows the dichroism of the pre-edge region assigned to the 1s-to-3d transition. F/I_0 , absorbance; E , energy. (B) FTs of polarized Mn EXAFS spectra from single crystals of PSII in the S_1 state. The FTs are from EXAFS spectra with the x-ray $\vec{e}$ -field vector aligned parallel to the crystal unit cell axes of PSII [a (red curve), b (blue curve), and c (green curve)]. Each of the three FT peaks characteristic of Mn EXAFS from PSII is dichroic. FT peak I is from Mn-ligand backscattering; FT peak II is from three Mn-Mn distances at 2.7 to 2.8 Å; and FT peak III is from one Mn-Mn and two Mn-Ca distances at 3.3 and 3.4 Å, respectively. All Fourier peaks appear at an apparent distance R' that is shorter than the actual distance R by ~ 0.5 Å due to a phase shift. The dichroism of the metal-to-metal distances reflects the geometry of the Mn_4Ca cluster.

Fig. 2.

(**A**) Comparison of Mn XANES spectra from solution PSII samples in the S_1 state (*T. elongatus*) (green curve) with the powder spectrum (red curve) calculated from the three PSII single-crystal, polarized spectra (Fig. 1A). The corresponding second derivatives of the Mn XANES (bottom) reveal that the XANES spectra of the solution and the calculated powder spectra from polarized PSII single-crystal spectra are indistinguishable. (**B**) Comparison of the FTs of the Mn EXAFS spectrum from solution PSII samples (green curve) and the powder spectrum (red curve) calculated from the three PSII single-crystal, polarized EXAFS spectra (Fig. 1B). The FTs are indistinguishable within the signal-to-noise ratio.

Fig. 3.

High-resolution structural models for the Mn_4Ca cluster in PSII from polarized EXAFS. The Mn_4Ca models I, II, and III are shown on the right. Each model is compatible with the polarized Mn EXAFS spectra of single crystals of PSII. The Mn_4 motif (**r** in fig. S1) common to the three structures is shown at upper left. The models are shown in the orientation in which they should be placed in the PSII membrane according to the axis system shown at upper right. The z axis is chosen to be along the membrane normal. The x and y axes define the membrane plane; the x axis connects the two nonheme Fe atoms of the dimer that are related by the noncrystallographic local C_2 axis. The particular orientations shown for models I, II, and III were chosen on the basis of their compatibility with the overall electron density and the positioning of the protein ligands in the 3.0 Å resolution x-ray crystal structure (16). The Mn atoms are shown in red. The distance between Mn_C and Mn_D atoms is ~ 2.8 Å (indicated by blue oxo bonds), and the distance between the Mn_A and Mn_B atoms, as well as the Mn_B and Mn_C atoms, is ~ 2.7 Å. The distance between Mn_B and Mn_D is ~ 3.3 Å. The Ca atom (green sphere) is ~ 3.4 Å from two Mn atoms [in model II, Ca can be at ~ 3.4 Å to Mn_D and Mn_C or Mn_D and Mn_B (model IIa, fig. S5)]. The bridging motif to Ca is not well defined by our experiments; therefore, dashed lines connect the Ca atom to the two Mn atoms at ~ 3.4 Å. The experimental FTs from the polarized Mn EXAFS spectra from single crystals (Fig. 1B) are comparable to the FTs calculated for these models. The dichroism of the EXAFS spectra will be identical for local C_2 -related orientations.

Fig. 4. Placement of model II for the Mn_4Ca cluster derived from polarized Mn EXAFS in relation to the putative ligands obtained from the 3.0 Å resolution x-ray crystal structure (16). The spheres represent Mn (red), Ca (green), and the bridging oxygen ligand atoms (gray). The assignment of ligands is tentative because it is based on the electron density of the Mn_4Ca cluster, and its immediate environment may be altered by x-ray damage. (A) Stereo view of the electron density (16) with model II placed in the density, as explained in the text. The view is along the membrane plane. (B) Same view as in (A) with ligands and atoms labeled correspondingly. All ligands are from D1 except for CP43-Glu³⁵⁴. Electron density was omitted for clarity. (C) A schematic diagram of model II with the view along the membrane plane, with putative ligands from the electron density. Bonds between Mn and the bridging oxo are shown as solid green lines. Bonds to putative terminal ligand atoms from amino acids and to Ca atoms are shown as dotted lines (black, less than 3.0 Å; blue, more than 3.0 Å).