X-ray damage to the Mn$_4$Ca complex in single crystals of photosystem II: A case study for metalloprotein crystallography


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X-ray absorption spectroscopy was used to measure the damage caused by exposure to x-rays to the Mn$_4$Ca active site in single crystals of photosystem II as a function of dose and energy of x-rays, temperature, and time. These studies reveal that the conditions used for structure determination by x-ray crystallography cause serious damage specifically to the metal-site structure. The x-ray absorption spectra show that the structure changes from one that is characteristic of a high-valent Mn$_4$(H$_2$O)$_4$ oxo-bridged Mn$_4$Ca cluster to that of Mn(II) in aqueous solution. This damage to the metal site occurs at a dose that is more than one order of magnitude lower than the dose that results in loss of diffraction power and is commonly considered safe for protein crystallography. These results establish quantitative x-ray dose parameters that are applicable to redox-active metalloproteins. This case study shows that a careful evaluation of the structural intactness of the active site(s) by spectroscopic techniques can validate structures derived from crystallography and that it can be a valuable complementary method before structure-function correlations of metalloproteins can be made on the basis of high-resolution x-ray crystal structures.

Oxygen, which supports all aerobic life, is abundant in the atmosphere because of its constant regeneration by photosynthetic water oxidation by green plants and cyanobacteria. The structural changes of the catalytic Mn$_4$Ca complex that generates oxygen by the oxidation of water has been the subject of intense study ever since Mn was identified as an essential element (1–4). The structural features of the Mn complex have been studied since Mn was identified as an essential element (1–4). The structural features of the Mn complex have been studied since Mn was identified as an essential element (1–4).

The Mn XANES data from PS II single crystals show that under the conditions of the recently published x-ray diffraction measurements, the Mn is largely reduced to Mn(II) from Mn$_4$(H$_2$O)$_4$ present in the native S$_1$ state cluster. Moreover, the EXAFS spectrum changes significantly from one that is characteristic of a high-valent multinuclear oxo-bridged Mn$_4$Ca cluster to one that is typical of mononuclear hexacoordinated Mn(II) in solution.

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Abbreviations: EXAFS, extended x-ray absorption fine structure; PS II, photosystem II; XANES, x-ray absorption near edge spectroscopy; FT, Fourier transform.

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The common unit for dose used in x-ray spectroscopy is photon density or photons per unit area at a given energy. The unit used in crystallography is the gray (J/kg). We show both of these units in Fig. 2, for purposes of comparison. A common dose limit for crystallography studies is $2 \times 10^{10}$ photons (25), which, at 6.6 keV, corresponds to $1 \times 10^{10}$ photons per $\mu$m$^2$.

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Materials and Methods

PS II crystal samples from *Thermosynechococcus elongatus* were prepared as described by Kern et al. (33). The PS II solution samples were prepared at a concentration of $5 \text{ mM chlorophyll a}$ by dissolving crystals in a buffer containing $50\%$ glycerol, $50 \text{ mM Pipes-NaOH (pH 7.0)}$, $3 \text{ mM CaCl}_2$, and $0.015\% n$-dodecyl-$\beta$-D-maltoside.

X-Ray Dose for Diffraction Studies. The average total number of x-ray photons per area or dose that each segment of PS II crystal was exposed to during typical x-ray diffraction studies at the three synchrotron radiation sources by three different groups are as follows: (i) European Synchrotron Radiation Facility, beamline ID 14-2, $2.3 \times 10^{10} \text{ photons per } \mu \text{m}^2$; (ii) SPring-8 (Hyogo, Japan), beamline BL41XU, $1.8 \times 10^{10} \text{ photons per } \mu \text{m}^2$; and (iii) Swiss Light Source (Villigen, Switzerland), beamline S065A, $1.75 \times 10^{10} \text{ photons per } \mu \text{m}^2$. The exposure was at $100 \text{ K}$, and all XANES was collected at $10 \text{ K}$ at low dose ($1 \times 10^7 \text{ photons per } \mu \text{m}^2$). XANES and difference spectra show that the increase in amplitude at $6,552 \text{ eV}$ provides definite evidence for the photoreduction to Mn(II) in PS II crystals by exposure to x-rays (see C). At a dose of $2.3 \times 10^{10} \text{ photons per } \mu \text{m}^2$, equal to $66\%$ of the average dose used for diffraction measurements, $80\%$ of the Mn in PS II is present as Mn(III). (B) A similar trend in the XANES (Upper) and the difference spectra (Lower) is seen for PS II solutions. The x-ray doses used for exposure were $0.03, 0.05, 0.10, 0.17, 0.32, 0.76,$ and $1.4 \times 10^{10} \text{ photons per } \mu \text{m}^2$ (light-blue to black lines). At a dose of $1.4 \times 10^{10} \text{ photons per } \mu \text{m}^2$, equal to $40\%$ of the average dose used for diffraction measurements, $90\%$ of the Mn in PS II is present as Mn(II). (C) The changes in the XANES spectra from an intact PS II sample as a function of addition of $10\%$ increments of the XANES spectrum of aqueous Mn(II) (Upper) and the corresponding difference spectra (Lower) (light-blue to black lines). The similarity between the difference spectra in A, B, and C is striking, showing the photoreduction of the Mn(III,IV) in native PS II to Mn(II). (D) A comparison of Mn K edge spectra from two tetranuclear complexes, $[\text{Mn}_4\text{O}_{3}\text{(OAc)}_4\text{(dbm)}_3]_4$ (35, 36) and $[\text{Mn}_2\text{O}_2(\text{tphpn})_2]\text{[ClO}_4\text{]}_4$ (37, 38), in oxidation states Mn(III,IV) and Mn(II) similar to those in intact PS II and from Mn(II) in aqueous solution.

![Fig. 1. Mn XANES of PS II versus x-ray dose and XANES of inorganic model compounds. (A) Mn K edge shift of PS II crystals as a function of x-ray dose at 13.3 keV (0.933 Å) and 100 K (Upper) and the difference spectra (Lower). The spectrum at the highest inflection point energy is from an undamaged PS II crystal. The x-ray doses used for exposure are 0.14, 0.21, 0.25, 0.54, 0.95, 2.3, and $5.0 \times 10^{10} \text{ photons per } \mu \text{m}^2$ (light-blue to black lines). An average dose of $3.5 \times 10^{10} \text{ photons per } \mu \text{m}^2$ was used for x-ray diffraction studies. The exposure was at 100 K, and all XANES was collected at 10 K at low dose ($1 \times 10^7 \text{ photons per } \mu \text{m}^2$). XANES and difference spectra show that the increase in amplitude at $6,552 \text{ eV}$ provides definite evidence for the photoreduction to Mn(II) in PS II crystals by exposure to x-rays (see C). At a dose of $2.3 \times 10^{10} \text{ photons per } \mu \text{m}^2$, equal to $66\%$ of the average dose used for diffraction measurements, $80\%$ of the Mn in PS II is present as Mn(II). (B) A similar trend in the XANES (Upper) and the difference spectra (Lower) is seen for PS II solutions. The x-ray doses used for exposure were $0.03, 0.05, 0.10, 0.17, 0.32, 0.76,$ and $1.4 \times 10^{10} \text{ photons per } \mu \text{m}^2$ (light-blue to black lines). At a dose of $1.4 \times 10^{10} \text{ photons per } \mu \text{m}^2$, equal to $40\%$ of the average dose used for diffraction measurements, $90\%$ of the Mn in PS II is present as Mn(II). (C) The changes in the XANES spectra from an intact PS II sample as a function of addition of $10\%$ increments of the XANES spectrum of aqueous Mn(II) (Upper) and the corresponding difference spectra (Lower) (light-blue to black lines). The similarity between the difference spectra in A, B, and C is striking, showing the photoreduction of the Mn(III,IV) in native PS II to Mn(II). (D) A comparison of Mn K edge spectra from two tetranuclear complexes, $[\text{Mn}_4\text{O}_{3}\text{(OAc)}_4\text{(dbm)}_3]_4$ (35, 36) and $[\text{Mn}_2\text{O}_2(\text{tphpn})_2]\text{[ClO}_4\text{]}_4$ (37, 38), in oxidation states Mn(III,IV) and Mn(II) similar to those in intact PS II and from Mn(II) in aqueous solution.](pnas.0505207102)
using a much lower x-ray dose than that typically required for high-resolution diffraction experiments (25). The x-ray dose and beam size sufficient for collecting XANES and EXAFS data are far less stringent than those required for the diffraction experiments. Moreover, all XAS experiments were performed at 10 K, compared with the 100 K that is normally used during crystallography. It is thereby possible to study the effect of x-rays on the Mn$_4$Ca cluster without inflicting any additional changes to its structure or oxidation state.

The methodology used for the collection of Mn XANES and EXAFS data and analysis is described in detail in refs. 32 and 34. These studies were carried out on beamline 9-3 and 10-2 at Stanford Synchrotron Radiation Laboratory and at the BioCAT 18ID beamline at Advanced Photon Source (Argonne, IL).

Aligning optics were optimized to produce intensity profiles that were plateaus without the presence of hot spots in the beam profile, slits were used to produce beams of precise shape, and filters were used to obtain beams of requisite dose. The sample was accurately positioned and scanned in the x-ray beam by using precision position encoders and a very low dose of photons (~1 x 10$^7$ photons per μm$^2$) in order not to cause any damage to the samples. The XAS spectra from single crystals are dichroic. Therefore, all spectra were collected at a specific orientation of the crystal to the x-ray e vector. The x-ray flux was measured by using a calibrated N$_2$-filled ion chamber placed before the sample. The XANES and EXAFS were collected as fluorescence excitation spectra by using a 30-element energy-discriminating Ge solid-state detector (Canberra, Meriden, CT). The diffraction pattern from the crystals was collected in situ by using a Mar 345 detector and was used for indexing the crystals before and after exposure to a given number of x-ray photons.

The following protocol was used for measuring the damage to the Mn$_4$Ca cluster in PS II crystals and solution samples. For each sample, first a XANES spectrum was collected at low dose (1 x 10$^7$ photons per μm$^2$) at 10 K to ascertain the intactness of the samples. The samples were then exposed to x-rays at 13.3 keV (0.933 Å), the wavelength used for native and heavy-atom derivative diffraction measurements, or at 6.6 keV (1.89 Å), the wavelength used for anomalous diffraction measurements. This exposure was performed at 100 K or at 10 K. X-ray dose and the time required for depositing the dose were varied. The sample was then immediately returned to 10 K if it was at 100 K, and the XANES and/or EXAFS (at a low dose of 1 x 10$^7$ photons per μm$^2$) was collected. The XANES spectrum from successive scans was invariant during these measurements. A new sample was used for each exposure to a particular x-ray dose, which ranged from 0.03 x 10$^{10}$ to 5 x 10$^{10}$ photons per μm$^2$. The x-ray dose was chosen to cover the range typically used at third-generation synchrotron sources and, more specifically, to coincide with those used for all PS II diffraction studies (see above). Experimental conditions, such as x-ray flux of the beamline, exposure time per frame, and beam size, are different for each of the three studies described above (see Supporting Materials and Methods, which is published as supporting information on the PNAS web site). Despite differences in experimental conditions, the doses for all of the studies are in a comparable range, and the representative dose for high-resolution data is ~3.5 x 10$^{10}$ photons per μm$^2$ or 1.6 x 10$^7$ grays (at 13.3 keV). In this study, the exposure dose is expressed in units of photons per area and in gray or relative to the representative value for current PS II diffraction studies.

**Quantitation of Mn(II) with XANES.** XANES spectra from a Mn(II) aqueous sample were added to that from an intact PS II sample in different proportions (Fig. 1C) and compared with the XANES spectra obtained from single-crystal and solution PS II samples that had been exposed to a specific dose of x-rays (Fig. 1 A and B). A direct comparison usually was sufficient to obtain the percentage of Mn(II) present in such samples. We also used the difference spectra (Fig. 1 A, B, and C) and the second derivatives (not shown) of the spectra for obtaining values with an accuracy of ±5% Mn(II).

**Results and Discussion**

Fig. 1A shows the Mn XANES data collected at 10 K from single crystals subsequent to exposure to a specific dose of 13.3-keV x-rays at 100 K; the typical published x-ray diffraction data of PS II crystals were collected by using 13.3-keV x-rays at 100 K. The Mn XANES spectrum of the intact PS II sample (red line, Fig. 1A) has the highest inflection point energy and compares well to the XANES from Mn model compounds in mixed-valent III,IV oxidation states (Fig. 1D) (35–38). It is also well established from several other studies that the Mn$_4$Ca complex in the oxygen-evolving complex of PS II is present in oxidation states (III,IV$_2$) in the native S$_1$ state (5, 34, 39, 40). The spectral features change significantly, along with the shift to lower energy, as the x-ray dose increases from 0.14 x 10$^{10}$ to 2.3 x 10$^{10}$ photons per μm$^2$ (4–66% of the average dose of 3.5 x 10$^{10}$ photons per μm$^2$ used for x-ray diffraction studies), and the XANES spectrum of the PS II crystals begins to resemble that of Mn(II).

![Fig. 2. Increasing Mn(II) content in PS II due to radiation damage. (Solid blue line) Mn(II) content in PS II crystals as a function of x-ray irradiation at 13.3 keV (0.933 Å). The irradiation was carried out at 100 K. The conditions are similar to those during x-ray diffraction data collection. The dose on the x-axis is given in gray and in photons per unit area, units that are commonly used for crystallography and spectroscopy experiments, respectively. The points on the curve represent samples that received between 0.14 and 5.0 x 10$^{10}$ photons per μm$^2$. At 66% of the dose (2.3 x 10$^{10}$ photons per μm$^2$) compared with the representative average dose (3.5 x 10$^{10}$ photons per μm$^2$) used for crystallography, PS II crystals contain ~80% Mn(II). (Dashed blue line) The damage profile for PS II solution samples is very similar to that seen for crystals, although it is slightly higher for the same dose. EXAFS spectra of samples shown in Fig. 3 are at levels of damage denoted on the right by A, B, C, D, and E. (Dashed green line) The generation of Mn(II) is considerably greater when the x-ray irradiation is at 6.6 keV (1.89 Å), which is the energy at which the anomalous diffraction measurements for PS II were conducted. Solid blue line) The Mn(II) produced by damage in crystals is considerably decreased when the irradiation is conducted at 10 K, providing a method that could be used to mitigate the effects of radiation damage during crystallography measurements.](image-url)
of aqueous Mn(II) (black line) shown in Fig. 1D. A similar trend is observed for PS II solution samples as shown in Fig. 1B. The damage to the crystals is also independent of the mosaicity of the crystals. The amount of Mn(II) present in the exposed S1 state samples is a measure of the x-ray-induced photoreduction of the Mn4Ca complex and can be quantitated as shown in Fig. 1C. XANES spectra are very sensitive markers of the amount of Mn(II) and have been used in several studies to quantify and establish the damage or contamination of native PS II with Mn(II) (41, 42).

The content of Mn(II) produced in the single crystals at 100 K as a function of the dose of 13.3-kV x-ray photons per mm2 is shown in Fig. 2 (solid blue line). There is a rapid rise in the generation of Mn(II), followed by a slower phase. Remarkably, even at 4% of the average dose used in crystallography, 15% of Mn in the crystal is reduced to Mn(II), and the generation of Mn(II) rapidly rises, reaching a value of 80% Mn(II) at 66% of the average dose. This result shows that, even at very modest doses, much of the Mn in the PS II crystals is photoreduced to Mn(II) during the diffraction studies.

The Mn(II) generated in PS II solution samples as a function of x-ray exposure follows the same trend seen in crystals (dashed blue line, Fig. 2). The solution samples are slightly more prone to damage; however, the differences are small. We can speculate that the damage is a function of the water content, because it is known that x-ray-induced damage can be mitigated by hydroxyl radicals (43). The damage and Mn(II) formation are significantly mitigated by lowering the sample temperature from 100 to 10 K during exposure (see Fig. 4, which is published as supporting information on the PNAS web site). The damage decreases by >50% for a similar dose (red line, Fig. 2), probably because of the decrease in the diffusion of the radicals generated by x-rays.

The damage to the Mn4Ca cluster as monitored by the generation of Mn(II) is much higher at the lower energies (1.89 Å, 6.6 keV, and 100 K), where the anomalous diffraction studies are carried out (dashed green line, Fig. 2). This increase is expected because the x-ray absorption cross section of the PS II matrix, mostly composed of C, N, and O atoms, is higher at 6.6 keV compared with the cross section at 13.3 keV (see Fig. 5, which is published as supporting information on the PNAS web site). These results illustrate the extreme difficulties of collecting anomalous diffraction data. The damage is even greater at 5.5 keV (2.25 Å), where one of the anomalous diffraction data sets was collected (16) to identify electron density from Ca in the Mn4Ca complex.

The time of exposure was not a critical parameter for Mn(II) generation at either 10 or 100 K for 13.3 or 6.6 keV x-ray energy...
decreases in amplitude, along with the appearance of a new peak characteristic of Mn–Mn interactions at 2.7 Å, also significantly mitigated. The bridged Mn4Ca complex with Mn–Mn–Mn–Ca, and Mn–Mn–Mn distances is disrupted and is replaced by Mn(II) that is similar to Mn(II) in solution. Moreover, this disruption begins to occur in a significant manner at a low x-ray dose (point A) and increases dramatically when the dose is increased (points B, C, and D). It is evident that the bridged Mn4Ca complex is absent at dose levels comparable to those used for x-ray diffraction. Apart from limited resolution (3.2–3.8 Å), the diffuse nature and the differences in the electron density among the four studies (14–17) probably result from a heterogeneous mixture of damaged Mn4Ca complex and aqueous Mn(II). The damage may also alter the positions of the putative Mn/Ca ligands (13, 20, 21). Hence, it is premature to either include or exclude proposed mechanisms for oxygen evolution on the basis of the current structural model from x-ray crystallography (16).

It is well recognized that radiation damage is a serious and inherent problem in x-ray crystallography, leading to a loss of resolution and an increase in mosaicity and the overall B factor (24, 43, 47, 48). Inspection of consecutive diffraction frames (see Fig. 6, which is published as supporting information on the PNAS web site) shows that such effects are significant for PS II crystals. Clearly, the effects of a high dose are not limited to decay of the diffracting power of the crystal, but highly specific changes leading to cleavage of disulfide bonds and decarboxylation of aspartic and glutamic acid residues occur at much lower dose (29, 47, 49). This mode of damage is especially true of solvent-exposed regions of proteins but also of active sites of enzymes, which, by their very nature, are accessible to water and therefore more susceptible to damage (24, 47). In proteins that contain redox-active metals, the reduction of the metal site and structural damage can be even more severe, as recognized for heme and Cu proteins two decades ago by Chance et al. (30, 50) and established quantitatively in this study. The Mn4Ca cluster should by its very nature be sensitive to x-ray radiation, because it is accessible to water molecules, and the Mn and Ca ions are coordinated predominantly by the carboxylate side chains of the protein.

It should be recognized that in the presence of redox-active metals, structural damage to the metal site could precede loss of diffractivity by two orders of magnitude of x-ray dose. This study provides ways for monitoring and decreasing the x-ray damage to the Mn4Ca complex in PS II and for other redox-active metalloenzymes. It is imperative that the position of the crystal is changed often and that more crystals are used for data collection. The temperature should be decreased from 100 to 10 K, because metal reduction and concomitant structural damage are thereby significantly mitigated. Finally, increasing the energy of x-rays from 6.6 (1.894 Å) to 13.3 keV (0.933 Å) significantly decreases radiation damage to the cluster; therefore, raising the energy further to 20–25 keV (0.6–0.5 Å) (24, 51) should be explored.
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