Rapid Loss of Structural Motifs in the Manganese Complex of Oxygenic Photosynthesis by X-ray Irradiation at 10–300 K*  

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Structural changes upon photoreduction caused by x-ray irradiation of the water-oxidizing tetramanganese complex of photosystem II were investigated by x-ray absorption spectroscopy at the manganese K-edge. Photoreduction was directly proportional to the x-ray dose. It was faster in the higher oxidized S2 state than in S1; seemingly the oxidizing potential of the metal site governs the rate. X-ray irradiation of the S1 state at 15 K initially caused single-electron reduction to S0 accompanied by the conversion of one di-µ-oxo bridge between manganese atoms, previously separated by ~2.7 Å, to a mono-µ-oxo motif. Thereafter, manganese photoreduction was 100 times slower, and the biphasic increase in its rate between 10 and 300 K with a breakpoint at ~200 K suggests that protein dynamics is rate-limiting the radical chemistry. For photoreduction at similar x-ray doses as applied in protein crystallography, halfway to the final MnII state, the complete loss of inter-manganese distances <3 Å was observed, even at 10 K, because of the destruction of µ-oxo bridges between manganese ions. These results put into question some structural attributions from recent protein crystallography data on photosystem II. It is proposed to employ controlled x-ray photoreduction in metalprotein research for: (i) population of distinct reduced states, (ii) estimating the redox potential of buried metal centers, and (iii) research on protein dynamics.

Numerous enzymes contain protein-bound metal centers forming their active site. A prominent example is the water-oxidizing manganese-calcium (Mn4Ca) complex of oxygenic photosynthesis bound to the D1 protein of photosystem II (PSII), which is embedded in the thylakoid membrane of higher plants, green algae, and cyanobacteria. The manganese complex catalyzes the light-driven oxidation of two water molecules, yielding reducing equivalents, protons, and the dioxygen of the atmosphere. By the sequential absorption of four quanta of visible light by PSII that drive the stepwise abstraction of four electrons, the manganese complex cycles through four semi-stable states called S1, S2, S3, and S0, where the subscripts denote the number of accumulated oxidizing equivalents (2). The S1 represents the dark-stable state; dioxygen is liberated only in the S3 → S0 transition (for reviews see Refs. 1 and 3).

Important structural information on the manganese complex has been obtained by x-ray absorption spectroscopy (XAS) (Refs. 4–7 and the references therein) and recently also by protein crystallography (8–11). The crystallographic results on PSII represent a long awaited breakthrough. With respect to the manganese complex, however, the question has emerged regarding to what extent the obtained structural information is invalidated by modifications caused by the numerous radicals that are inevitably created by x-ray irradiation (11–13). In all four structures (8–11) manganese ions were found; however, there were inconsistencies in their probable number and position with respect to the protein matrix. In addition, surprisingly few amino acid side chains were at a reasonably short distance to provide ligands (Fig. 1). Water molecules, which were invisible at the best resolution of 3.2 Å (11), or disorder effects may explain the void around manganese. As previously estimated (12), recently shown by XAS on PSII crystals (14), and substantiated in this work, all crystallographic data likely refer to a metal site where x-ray exposure resulted in reduction of the initially high valent manganese ions to the MnII level.

In protein crystallography, which is mostly done at 100 K at high flux third generation synchrotron radiation sources, prolonged x-ray irradiation of crystals eventually leads to a decrease in the diffracted intensity (15–17). Frequently, data collection is continued until this “radiation damage” becomes too pronounced for compensation by scaling of mosaicity and temperature factors. This approach becomes critical, if distinct modifications occur already prior to the loss in diffraction quality. Indeed, specific structural changes result from x-ray irradiation at ~13 keV (0.95 Å) even at low temperatures, e.g. breaking of disulfide bonds, modification of carboxylates and histidines, and tyrosine dehydroxylation, before the global fading out of diffraction intensity (18–20). Reduction of active site transition metal ions may precede modifications of the protein matrix (21, 22).

In the present study, the influence of ionizing x-ray irradiation on the manganese complex of PSII is investigated systematically by XAS at the manganese K-edge (23, 24). As opposed to crystallography, XAS is particularly sensitive to the oxidation state and local structure of protein-bound metal centers (6). The position on the energy scale of the K-edge in the XANES region of XAS spectra is indicative of the formal oxidation state of the metal, at least in the case of the manganese complex of PSII (see Ref. 6 and the references therein), and thus was employed to monitor the time course of the photoreduction process. The EXAFS spectral region, which carries information on the metal–ligand distances, on the number and chemical nature of ligands, and on the geometry of the site, e.g. as maintained by well known bridging motifs such as di- and mono-µ-oxo bridges between the metal ions, was scrutinized in detail to detect changes in the structure of the manganese complex.

The relations between x-ray flux and exposure time, temperature, and the rate and extent of manganese photoreduction and the associated structural changes were quantified. The implications of the results for interpretation of the crystallographic data are discussed. The obtained insights are of likely relevance for protein-bound metal centers in general and for the use of x-ray photoreduction as a specific tool to study intermediates of the catalytic cycle.

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2 The abbreviations used are: PSII, photosystem II; EXAFS, extended x-ray absorption fine structure; FT, Fourier transform; MES, 2-morpholinoethanesulfonic acid; XANES, x-ray absorption near edge structure; XAS, x-ray absorption spectroscopy.

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FIGURE 1. The four manganese ions in PSII and their surrounding amino acid residues in crystallographic data (11) at 3.2 Å resolution (Protein Data Bank entry 1W5C). Likely bonds to manganese from amino acid side chains are shown as thick lines (manganese-ligand distance, \( R < 2 \) Å), and possible bonds are shown as dotted lines (manganese-ligand distance, \( 2 < R < 3.5 \) Å). In the crystallographic model of Ref. 11, all of the other atoms in the structure are at distances of \( >3.5 \) Å from manganese. Excepting Glu\(^{354} (*) \) of the CP43 subunit, all of the amino acids belong to the D1 subunit of PSII.

FIGURE 2. Normalized K-edge spectra of the manganese complex of PSII measured at the low flux beam line at 20 K. 10 scans were averaged for each spectrum. Spectra of the dark-stable \( S_1 \) state (△) and the light-induced \( S_2 \) state (■) and of the states created by x-ray irradiation of \( S_1 \) samples for 18 h (S\(^0_1\) * , ▴) and of \( S_2 \) samples for 6 h (S\(^1_1\) *, □) are displayed.

MATERIALS AND METHODS

Sample Preparation and S-State Population—PSII-enriched membrane particles were prepared from market spinach using betaine as a stabilizer in all media as described in Refs. 25 and 26 and stored at \(-80^\circ\)C until use. Their oxygen evolution activity under saturating white light illumination was 1200–1400 m\( \text{O}_2 \) (mg chlorophyll \( \times h^{-1} \)) at 28 \( ^\circ \)C. PSII membrane particles were dissolved at 1 mg/ml of chlorophyll at 28 °C. PSII membrane particles were dissolved at 1 mg/ml of chlorophyll and partially dehydrated membrane multilayer samples on Kapton foil were prepared by the previously described centrifugation technique (25) and enriched in the \( S_1 \) state using a preflash protocol (7, 25). \( S_1 \) state samples were prepared by continuous white light illumination (440 nm \( \pm 20 \) nm) of \( S_1 \) samples for 2 min at the 200 K of a dry ice/ethanol bath (7, 25). Quantitative population of the \( S_2 \) state was verified by EPR measurements (not shown) of its well known multilinear signal (27).

XAS at Low Flux, Bending Magnet Beam Line—XAS measurements at the manganese K-edge were performed at the EMBL bending magnet beam line D2 at Hamburg Synchrotron Laboratory (Deutsches Elektronen-Synchrotron, Hamburg, Germany) using an energy-resolving solid state 13-element germanium detector (Canberra) for fluorescence detection (25). The Si111 crystal monochromator was detuned to 70% of maximal flux. The samples were kept in a helium cryostat at 20 K under \(-200 \) mbar of helium gas. The x-ray spot size on the samples was \( \approx 4 \times 1 \) mm. The scan duration (6.400–7100 eV) was \( \approx 20 \) min.

XAS at High Flux, Undulator Beam Line—XAS measurements at cryogenic temperatures as well as at room temperature were carried out at the undulator beam line ID26 of the European Synchrotron Radiation Facility (Grenoble, France). Manganese XAS spectra were measured by monitoring the excited x-ray fluorescence perpendicularly to the incident beam by a PIN photodiode (3.8-cm\(^2\) active area; EuriSys Measures), as in Refs. 6 and 28. In the room temperature experiments, the samples were exposed to plain air of the climatized (18 ± 2 °C) experimental hutch of the beam line. In the low temperature experiments, the samples were kept in a helium cryostat at \(-200 \) mbar of helium gas. Rapid scan XAS spectra were collected by simultaneous scanning of a Si220 crystal monochromator (scan range, 6500–7100 eV; x-ray spot size on the sample, \( \approx 1 \times 1 \) mm) and the undulator gap in the rapid scan mode of ID26 (29) as described in Refs. 28 and 30.

At both synchrotron radiation sources, the angle between the electric field vector of the incident x-ray beam and the sample normal was 55° (magic angle; see Ref. 31). Energy calibration of each XAS spectrum was facilitated by simultaneous measurements and Gaussian simulation of the narrow pre-edge peak absorption centered at 6543.3 eV of a KMnO\(_4\) powder sample mounted in front of a detector at the end of the beam line. The accuracy of the energy calibration procedure is better than ±0.1 eV (25, 28).

Data Evaluation—XAS spectra were normalized, and EXAFS oscillations were extracted and transformed to a wave vector scale (\( \tilde{k} \) scale) using an \( E_0 \) value of 6540 eV as described in Ref. 6. Edge energies were determined by the “integral method” (6). EXAFS oscillations were weighted by \( k^2 \) to compensate for the fall-off of amplitudes with increasing energy. Spectra were simulated by a least squares procedure using the in-house software SimX (32). Complex phase functions for different elements in the various shells of backscatters were calculated using FEFF-7 (33). The amplitude reduction factor, \( S_{00}^2 \), was 0.85. In the simulations a value for \( E_0 \) of 6547 eV was consistently used. The fit quality was judged by calculation of the Fourier-filtered R-factor (\( R_F \)) (28) subsequently to the curve fit itself, which involved only unfiltered EXAFS oscillations (6). Fourier transforms of EXAFS oscillations were calculated for energies between 20 and 500 eV above \( E_0 \) using fractional cosine windows extending over 10% at high and low ends of the \( k \) range.

RESULTS

X-ray Photoreduction of the Manganese Complex at 10–20 K—A series of consecutive XANES spectra was collected on one single spot on the PSII samples. These spectra reflect the radiation-induced modifications of the manganese complex, which gradually develop with increasing x-ray exposure time. To resolve the initial steps of the x-ray photoreduction of manganese at 20 K, spectra were collected at a low flux bending magnet beam line (D2, Hamburg Synchrotron Laboratory, Deutsches Elektronen-Synchrotron, Hamburg, Germany). The slower photoreduction steps became experimentally accessible only at an undulator beam line (ID26, European Synchrotron Radiation Facility, Grenoble, France), where the x-ray flux was \( \approx 3 \) orders of magnitude higher and photoreduction was accelerated accordingly.

Reduction of manganese typically results in a shift of its K-edge position to lower energies (6). Fig. 2 shows edge spectra of the manganese complex not previously exposed to X-rays for the dark-stable \( S_1 \) state and for the \( S_2 \) state, both measured at the low flux beam line at 20 K. The oxidation state of the Mn\(_{4+} \) complex in these states most likely is
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Mn_{III}^3Mn_{IV}^3 and Mn_{III}^3Mn_{IV}^3, respectively (Ref. 7 and the references therein). Accordingly, the light-induced S_1 \rightarrow S_2 transition is associated with a one-electron oxidation of the Mn_4 complex, which causes a shift of the K-edge by 0.5–0.9 eV to higher energies (7, 25, 34). Using the integral method (6), a similar upshift by \(-0.7\) eV as previously found (7) was obtained here (Fig. 2).

For samples in the more oxidized S_2 state, \(-6\) h of x-ray irradiation at the low flux beam line yielded a XANES spectrum, which resembles that of S_1 with respect to edge energy and shape (Fig. 2). The K-edge position at 20 K in the S_1 and S_2 state samples as a function of the x-ray exposure time is shown in Fig. 3. For samples initially in S_2 (triangles), it was confirmed that \(-6\) h of irradiation caused reduction by 1 equivalent of the Mn_4 complex as judged by the downshift of the K-edge energy by \(-0.7\) eV. On the other hand, for samples initially in S_1 (circles), \(-18\) h of irradiation yielded a downshift by \(-0.7\) eV (Fig. 3) to a state that resembled the native S_1 (7) with respect to its edge energy (Fig. 2). Thus, the initial photoreduction of the manganese complex in its higher oxidized S_2 state was more than two times faster than in S_1. We conclude that the velocity of x-ray photoreduction critically depends on the oxidizing power of the metal center, in line with previous results (7).

The slower steps of x-ray photoreduction were monitored by collecting successive XAS spectra at 10 K at the high flux beam line on S_1 state samples (Fig. 4B). A clearly biphasic decrease of the edge energy was observed. Its bi-exponential simulation yielded for the faster edge shift by approximately \(-0.7\) eV a time constant of \(\tau_\text{fast} = 1/k_\text{fast} = 1.1\) min. Comparison of the initial phase of photoreduction in Figs. 3 and 4 revealed its 600-fold acceleration at the high flux beam line, which is by a similar factor as the increase in the x-ray flux. The second main reduction phase accounting for most of the edge shift was described by \(\tau_\text{main} = 1/k_\text{main} = 110\) min. The rate constants are summarized in Table 1.

The edge energies in Fig. 4B were obtained from a series of complete XANES spectra. In Fig. 4C the edge energy was monitored by an alternative approach, namely the time scan technique (35). The x-ray fluorescence intensity was recorded at a fixed excitation energy of 6549.5 eV. At this energy, the x-ray absorption and thus the excited fluorescence increases for increasing reduction of the manganese complex (Fig. 4A). Fig. 4 (B and C) reveals similar kinetic behavior of the K-edge energy and of the increase in the x-ray fluorescence. Thus, the time scan approach can be used to monitor the K-edge energy of the metal and hence the level of x-ray photoreduction in an experimentally efficient way, which may also be employed at crystallography beam lines.

The pronouncedly biphasic downshift of the K-edge suggests the rapid formation of a discrete intermediate state upon the one-electron reduction of the manganese complex initially in S_1. In the water oxidation cycle, the light-driven oxidation of S_0 leads to S_1. Thus, a single reduction of S_1 leads to a formal S_0 state. Accordingly, the one-electron reduction of S_1 results in a formal S_0 state. We denote the intermediates created by x-ray photoreduction as S_0^* and S_1^*, respectively. The similar XANES spectra suggest a close correspondence between the native states (S_0 (7) and S_1) and the x-ray-induced intermediates (S_0^* and S_1^*). Notably, the more reduced states beyond S_0^* created by prolonged x-ray irradiation have no precedent in the native catalytic cycle.

Temperature Dependence of Photoreduction—Fig. 5 shows the results of x-ray photoreduction experiments at room temperature (290 K). Again, the irradiation time dependence of the edge shift did not exhibit a single-exponential behavior. Rather, as opposed to the rapid reduction phase observed at 10–20 K, at room temperature an initial lag phase was observed (Fig. 5A) as in Refs. 7 and 30. Accordingly, at room temperature the initial rate of photoreduction is close to zero, whereas it is particularly high at low temperatures. In the following, the initial phases were not considered but only the subsequent apparently monophasic reduction process, which accounts for the major fraction of the irradiation. 

![Figure 3: Decrease of the manganese K-edge energy during x-ray irradiation at the low flux beam line at 20 K of PSII samples initially in the S_1 ( ) and S_2 state ( ). The data points represent the K-edge energies of a series of XAS spectra recorded on the same sample spot each within 20 min. The x-ray flux was \(1 \times 10^{12}\) photons \(s^{-1} \cdot mm^{-2}\). An edge shift of \(-0.7\) eV indicated a one-electron photoreduction of the manganese complex in the S_1 \rightarrow S_0^* and S_2 \rightarrow S_1^* transitions with respective rate constants \(k_\text{fast} = 0.0014\) and \(0.0040\) min\(^{-1}\).](image3)

![Figure 4: Photoreduction at 10 K of the manganese complex in its S_1 state at the high flux beam line. A normalized manganese K-edge spectra for increasing x-ray exposure time. The x-ray flux was \(-0.5 \times 10^{12}\) photons \(s^{-1} \cdot mm^{-2}\). The arrow indicates the increase in the x-ray fluorescence level at an excitation energy of 6549.5 eV. B, decrease of the manganese K-edge energy derived from successive XAS scans of 30 s duration each on the same sample spot. Note the initial rapid decay of the K-edge energy leading to the S_0^* state. Biphasic simulation yielded rate constants of \(k_\text{fast} = 0.9\) min\(^{-1}\) and \(k_\text{main} = 0.009\) min\(^{-1}\). C, the x-ray fluorescence intensity excited at 6549.5 eV as function of the irradiation time.](image4)
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TABLE I
Dependence of photoreduction rates on x-ray flux and temperature for the manganese complex of PSII

| Initial S state | Beam line     | Flux (6.7 keV) | $k_{\text{fast}}^a$ | $k_{\text{main}}$ | Mn$^{114}b$ | T |
|----------------|---------------|----------------|---------------------|-------------------|-------------|---|
| $S_1$          | D2            | ~$1 \times 10^{11}$ | 0.0014              | ND                | (Weeks)     | 20 |
|                | ID26          | ~$0.1 \times 10^{12}$ | 0.5             | ND                | 20          |
|                |               | ~$1.0 \times 10^{12}$ | 4.9               | ND                | 20          |
|                |               | ~$0.5 \times 10^{12}$ | 0.9               | 0.009             | ~20,000     | 10 |
|                |               | ~$1 \times 10^{12}$ | ND                 | 0.020             | ~10,000     | 10 |
|                |               |                    | ND                 | 0.038             | ~5,000      | 100|
| $S_2$          | Crystallography$^c$ | ~$1 \times 10^{14}$ (~13 keV) | ~100               | ~1.0             | ~200        | 100|
|                | D2            | ~$1 \times 10^{12}$ | 0.0004             | ND                | (Weeks)     | 20 |

$^a$ $k_{\text{fast}}$ accounts for the formation of $S_0$.

$^b$ Estimated irradiation time for 90% reduction of the manganese complex to Mn$^{114}$.

$^c$ The applied dose in crystallography was 4 with rate $k_{\text{main}}$.

$^d$ Estimates for crystallography beam lines refer to fluxes reported in (Ref. 14) and to the considerations under “Discussion.” ND, not determined.

FIGURE 5. Photoreduction of the manganese complex at room temperature (290 K) at the high flux beam line. A, K-edge energies derived from successive XAS scans of 10-s duration on one spot of $S_1$ state samples; B, x-ray fluorescence time scan at an excitation energy of 6549.5 eV. Note the initial lag phase in the photoreduction process. The flux was ~$1 \times 10^{12}$ photons s$^{-1}$ mm$^{-2}$. Single exponential simulation of the major phase K-edge energy decrease in A yielded a rate $k_{\text{main}}$ of 0.9 min$^{-1}$.

FIGURE 6. Temperature dependence of the rate of manganese photoreduction. Data points (A) have been derived from single exponential simulations of x-ray fluorescence time scans (see inset) measured at the high flux beam line on $S_1$ state samples. The intersection of the straight lines marks the breakpoint at ~200 K in the increase of the rate with temperature.

FIGURE 7. X-ray fluorescence time scans measured at an excitation energy of 6549.5 eV on the manganese complex initially in its $S_1$ state (20 K, high flux beam line). A, maximal x-ray flux; B, x-ray flux attenuated to 10%. Note the 10-fold extended time axis in B.

Relation between X-ray Flux and Photoreduction Rate—The relation between the rate of photoreduction and the x-ray flux was investigated at the high flux beam line for PSII samples initially in the $S_1$ state by the time scan technique (Fig. 7). The time scans carried out at 20 K with maximal x-ray flux (Fig. 7A) or using an x-ray flux that was attenuated to 10% of the maximum (Fig. 7B) revealed identical biphasic behaviour, but a 10 times slower photoreduction in the latter case. Thus, a linear dependence of the reduction rate on the x-ray flux may be anticipated. To confirm this conjecture, fluorescence time scans at increasing flux were measured and simulated by Equation 1, taking into account the biphasic photoreduction at low temperatures,
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Reflect the presence of two di-µ-oxo-bridged manganese pairs in the $S_1$ state (Refs. 6, 7, and 12 and the references therein). (Deviating interpretations have been reported in Refs. 14 and 36.) Already 1.6 min of x-ray exposure prior to the EXAFS scan resulted in a significant decrease in the magnitude of peak III, suggesting a reduction in the number of the ~2.7-Å manganese-manganese vectors. After 5.5 min irradiation, peak III was diminished by ~50%. More prolonged x-ray irradiation resulted in the complete disappearance of peak III. In parallel, the formation of a new FT peak at ~1.8 Å (peak II) indicated the presence of manganese-ligand distances of ~2.2 Å. Such distances are typical for manganese-oxygen bonds in Mn$^{II}$ complexes (37). After very long x-ray irradiation, the peak II attributed to Mn$^{II}$-O interactions became the only detectable one (not shown) so that the spectrum resembled that of [MnII(H$_2$O)$_6$]$^{2+}$ in solution (14, 38).

Interestingly, there was a pronounced FT peak at ~3 Å that increased in size during the early phase of photoreduction and disappeared after longer irradiation times (Fig. 9B, asterisks). The transient increase of this peak likely reflects the appearance of a longer manganese-manganese vector of ~3.5 Å, presumably because of the formation of a mono-µ-oxo-bridged manganese pair in $S_1$ (38). Thereafter, all manganese-manganese vectors gradually became elongated to ~3.5 Å and thereby EXAFS-invisible during photoreduction to the final Mn$^{II}_4$ state.

To extract quantitative structural information, the $k^3$-weighted EXAFS spectra (Fig. 9A) were simulated as previously described (6). We focused on the manganese-oxygen/nitrogen and manganese-manganese distances in the first and second coordination spheres. Therefore, all backscatterers at distances above 3 Å were not considered in the curve fitting. This distance range has been shown to comprise manganese-manganese and manganese-calcium vectors of ~3.1–3.4 Å length (4–7, 14, 39). Their neglect may result in a minor increase of the apparent coordination number (from ~1.0 to ~1.25) of the ~2.7 Å shell assignable to di-µ-oxo-bridged manganese pairs (6, 38). Otherwise, however, the simulation results for the manganese-backscatterer shells below 3 Å remained essentially unaffected. The simulation curves obtained for a joint fit (38) of the nine spectra, including two shorter manganese-oxygen interactions (backscatter shells I and II) to account for the complex distance distribution function of the manganese-oxygen/nitrogen bonds (6) and one ~2.7 Å manganese-manganese vector.
manganese-manganese distance \( (R_{\text{Mn}}) \) changed only marginally, but its apparent coordination number \( (N_{\text{Mn}}) \) was reduced by \( \sim 50\% \). If it is assumed that there are two 2.7-Å manganese-manganese vectors in the \( S_0 \) state assignable to di-μ-oxo-bridged manganese pairs, seemingly after only \( \sim 5 \) min of irradiation one bridge was lost or modified. Dissolution of the second di-μ-oxo bridge during photoreduction was clearly slower but essentially completed after \( \sim 60 \) min. Then, as judged by the K-edge energy (Fig. 4) and by the coordination numbers of the manganese-oxygen/nitrogen vectors (Fig. 10A), the manganese complex was reduced by \( \sim 4 \) equivalents, presumably to the \( \text{Mn}^{III}_2\text{Mn}^{II}_3 \) state. Thus, already after approximately half of the irradiation time required to reduce all four manganese ions to \( \text{Mn}^{II} \), di-μ-oxo bridges seem to be absent in the complex.

In conclusion, even at temperatures as low as 10 K, x-ray-induced photoreduction of the manganese complex is coupled to the loss of most or all μ-oxo-bridging motifs between manganese ions. On the other hand, controlled photoreduction may be employed to investigate distinct intermediates as the \( S_0^* \) and \( S_1^* \) state, which likely are related to the native structure of the complex.

**DISCUSSION**

**X-ray Photoreduction: Mechanistic Considerations**—The PSII manganese complex initially is more than twice as rapidly reduced when starting from the \( S_2 \) state than from \( S_1 \) (Ref. 7 and this work). But also the \( S_1 \) state is reduced faster than metal sites of other enzymes (see below). At low temperatures, \( S_1 \) state first undergoes a rapid one-electron reduction resulting in \( S_0^* \). Further reduction to the \( \text{Mn}^{IV}_4 \) level is 100 times slower.

These results are in accord with the estimated redox potential of the manganese complex. In \( S_1 \), it is highly positive; approximately \(+1 \) V (41). A potential increase upon the \( S_1 \) to \( S_0 \) transition and a decrease in the \( S_0^* \) state is anticipated (41, 42); the potential of the \( \text{Mn}^{III}_4 \) state presumably is comparably low \((\pm 0 \) V) (43, 44). That electrochemical centers are the primary target for reduction by electrons liberated because of x-ray exposure is also supported by investigations on protein crystals where disulfide bridges first became reduced at 100 K (18, 19) and by EPR results (Ref. 45 and the references therein). We conclude that the high susceptibility of the PSII manganese complex to photoreduction is governed by its extraordinary positive potential.

Whereas the oxidation state of the metal center frequently can be determined from the energy of the metal K-edge, the redox potential of buried protein-bound metal centers mostly remains obscure. Quantification of the relation between electrophilicity and photoreduction rate could provide an estimate of the redox potential also for deeply buried metal sites.

Interestingly, the temperature dependence of the main rate of photoreduction of the manganese complex showed a sharp breakpoint at \( \sim 200 \) K. A similar breakpoint was observed in recent measurements of the mean square displacements, \( \Delta x^2 \), of the atoms in PSII membrane particles by quasi-elastic neutron scattering (46). Also in other proteins where \( (\Delta x^2) \) was studied for bound iron (47, 48), hydrogen atoms (49, 50), methyl-bearing side chains (51), and specific amino acid residues and associated water molecules (52), a breakpoint consistently was found at \( \sim 200 \) K. Similar breakpoint behavior was also observed for certain electron transfer steps in photosystem I (53) and in the bacterial photosynthetic reaction center (54).

The sharp increase of \( (\Delta x^2) \) above 200 K has been attributed to the onset of inharmonic, protein-specific dynamics. The breakpoint temperature may reflect the transition between two regimes of distinctly different dynamic behavior; occasionally the term "protein-glass transi-
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The observed linear relation between x-ray flux and the photoreduction rate of the manganese complex, suggesting that the x-ray dose determines the extent of photoreduction. Good evidence for a linear relation between flux and rate of radiation damage relation also has been obtained for protein crystals irradiated at 100 K (57), but also deviations from linear behavior were reported (58). Linearity in case of the PSII manganese complex implies that it is straightforward, at least in this system, to adjust either the x-ray flux or the irradiation time so that a crystallographic structure of the unperturbed manganese complex in its $S_1$ state may be reachable.

In the used PSIIL samples there are $\sim 10^5$ light atoms (carbon, nitrogen, and oxygen) per manganese complex (hydrogen atoms can be neglected because of their small absorption cross-section). At energies above the manganese K-edge (e.g. 6700 eV), their x-ray absorption cross-section, $\mu_{\text{C,N,O}}$ is roughly 40 times smaller than $\mu_{\text{Mn}}$ (59). Thus, less than 0.2% of the x-ray photons are absorbed by manganese. Also in absolute terms, direct absorption by manganese is a rare event as even at the sample surface; only $\sim 1.5\%$ of the manganese ions are hit during 1 h of irradiation at $10^{12}$ photons s$^{-1}$ mm$^{-2}$. Thus, the x-ray absorption by manganese ions is excluded as the primary cause of radiation-induced modifications.

Below $\sim 15$ keV the dominant primary event after x-ray absorption is photoionization. Mostly core electrons of carbon, nitrogen, and oxygen are liberated, causing a cascade of secondary ionization events, which results in the formation of electron-defficient cation radicals, electron surplus anion radicals, and population of excited states (45, 60). Assuming that less than 25 eV is required per ionization event, we estimate that more than 250 radical pairs are initially formed per absorbed x-ray photon in PSII. A significant fraction of these radicals is stable at temperatures $<100$ K (61).

The photoreduction is proportional to the accumulated dose but independent of the dose rate. This shows that thermally activated radical migration or chemistry proceeding on the seconds to minutes time scale is not rate-limiting for photoreduction. We propose that the initially formed excited radical states represent, prior to thermalization, the source for electron transfer to the manganese complex (electron sink). The electron transfer rate is determined by their distance and potential difference to the manganese complex and by the temperature (62). Electron transfer and possibly also hydrogen atom transfer and more complex chemistry competes with thermal and radiative (luminescence) excited state decay. Thus, thermal activation behavior comitant to dose-rate independence of x-ray photoreduction is predicted.

The $S_0^*$ State Created by X-ray Irradiation—X-ray irradiation of proteins has been used to reduce their metal site so that putative intermediates of the catalytic cycle became accessible for structure determination (21, 63). Underlying was the assumption that controlled irradiation generates distinct redox states of the metal before significant radiation damage to the protein occurs. We obtained evidence that this rationale also holds true for the PSII manganese complex.

Starting in the $S_0$ state, a rapid one-electron photoreduction leads to the formation of $S_0^*$. Accompanying is a reduction in the number of 2.7 Å manganese-manganese vectors by $\sim 50\%$, suggesting that one of

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Interestingly, also for the native $S_0$ state, a decrease in the apparent number of 2.7 Å manganese-manganese vectors relative to $S_1$ has been observed, but in this case individual manganese-manganese distances of 2.7 and 2.8–2.9 Å were resolvable (4, 7, 36). Thus, the native $S_0$ as created by light flash application at room temperature may contain one Mn(µ-O),Mn and one Mn(µ-O)(µ-OH)Mn unit (4, 7, 36). At room temperature the $S_0$ $\rightarrow$ $S_1$ transition is electronneutral (65, 66); probably the manganese oxidation is coupled to deprotonation of a µ-OH bridge (7, 36, 42). At the cryogenic temperatures where the $S_1$ $\rightarrow$ $S_0^*$ step occurred, charge-compensating long-range proton movements are unlikely so that protonation is impaired, and instead the µ-O bridge is broken.

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Recent crystallographic data suggested that one specific manganese ion ($Mn_I$ in Fig. 1), presumably coordinated by D1-Asp$^{170}$ is the first to become disordered after prolonged x-ray exposure of PSII. It is tempting to speculate that $Mn_I$ is connected to a neighboring manganese by a di-µ-oxo bridge in the $S_0$ state but by a mono-µ-oxo bridge in the $S_0^*$. Proton release from the µ-OH bridge upon the native $S_0$ $\rightarrow$ $S_1$ transition may then occur via the proton channel close to Asp$^{170}$, which has been
tentatively assigned in the PSII structure (10). Upon its reduction to MnII, the mono-μ-oxo bridge may be lost so that MnII becomes increasingly disordered. We summarize these attributions in Fig. 11.

Implications of Manganese Photo reduction for Crystallography—In protein crystallography at synchrotron radiation sources, data frequently are collected using x-rays of an energy E of ~13 keV. The x-ray absorption coefficient, μ(E), of carbon, nitrogen, and oxygen matrix atoms to a first approximation is proportional to 1/E², whereas the deposited energy per photon increases linearly with energy. Thus, for identical photon flux the photoreduction rate should decrease quadratically with increasing x-ray energy. Therefore, for crystallographic data collection at ~13 keV, the rate of photoreduction may be approximately four times lower than that reported here for irradiation at ~6.7 keV. This estimate is in line with previous experimental results (14).

In the present study, photoreduction of the manganese complex was investigated for a specific type of PSII preparation, namely multilayers of membrane particles in which the water content was reduced to ~50%. An increased water content (use of frozen solutions) or a decreased lipid content (in PSII core particle preparations used for crystallization) (14) and also stabilizers such as betaine and cryoprotectants (glycerol and sucrose) may slightly affect the photoreduction rate. In any event, on the basis of the results of the present study (Table 1), our previous estimates (12), and recent XAS experiments on PSII crystals (14), we conclude that likely all manganese ions became reduced to the MnIII level in crystallography at 100 K and using X-rays of ~13 keV.

To what extent is the crystallographic structure of the manganese complex affected by x-ray photoreduction? In crystallography at 100 K involving x-ray fluxes of ~10¹⁴ s⁻¹ mm⁻² (12, 14), the S₈ state certainly is rapidly reached (within a few seconds). In particular this rapid initial phase of photoreduction may easily be overlooked and indeed has not been noticed previously (14). Already in the S₈ state where only a single manganese ion is reduced by one equivalent, one di-μ-oxo bridge between manganese ions is lost, and the respective ~2.7 Å manganese-manganese distance is elongated to ~3.5 Å. More prolonged irradiation results in progressive MnIII formation and in the total loss of μ-oxo bridges between manganese ions so that all manganese-manganese distances become elongated even at 10 K. It is important to note that the loss of di-μ-oxo bridges was already completed halfway to reduction to the final MnIV state. Thus, in the crystallographic electron densities, manganese-manganese distances <3 Å presumably are no longer present, and the arrangement of the MnIII ions is pronouncedly different from the intact complex.

It is an unexpected result of this and previous (14) studies that the final MnIV state created at low temperatures spectroscopically resembles the [MnIV(H₂O)₆]²⁺ complex in solution. A straightforward interpretation of this observation is that not only μ-oxo bonds are broken but that also rearrangements of the manganese ions and of their ligands from amino acids occur so that additional H₂O molecules may become bound directly to MnIII. At the current crystallographic resolution of 3.2 Å (11), the displacement of manganese ions and amino acid side chains by <1 Å may be hard to detect. However, such structural changes could explain the unassigned space around manganese ions and the inconsistencies with respect to side chain orientations among the crystal structures (8–11).

A distinct structural model including a Mn₃Ca(μ₃-O)₄ cubane cluster plus a nearby “monomeric” manganese ion has been proposed (10). This model represents a stimulating hypothesis, but its specific features are likely to be revised if photoreduction during crystallography is decreased by more optimized experimental strategies. Preliminary evidence for a different position of the MnIV ion (Fig. 11) in crystal structures obtained at 100 and 20 K has been derived. Specifically, we propose that the apparently monomeric manganese ion may originally be connected to the complex by a di-μ-oxo bridge in the S₄ state but by a mono-μ-oxo bridge in the irradiation-induced S₈ state.

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Note Added in Proof—Recent crystallographic data suggest monodentate ligation of Asp-170 and Glu-333 to Mn in Fig. (1) (Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Nature, 438, 1040–1044). These and other differences in the ligation mode of manganese do not affect the results presented here.

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