Crystallographic and Spectroscopic Studies of Peroxide-derived Myoglobin Compound II and Occurrence of Protonated Fe$^{IV}$–O$^*$

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High resolution crystal structures of myoglobin in the pH range 5.2–8.7 have been used as models for the peroxide-derived compound II intermediates in heme peroxidases and oxygenases. The observed Fe–O bond length (1.86–1.90 Å) is consistent with that of a single bond. The compound II state of myoglobin in crystals was controlled by single-crystal microspectrophotometry before and after synchrotron data collection. We observe some radiation-induced changes in both compound II (resulting in intermediate H) and in the resting ferric state of myoglobin. These radiation-induced states are quite unstable, and compound II and ferric myoglobin are immediately regenerated through a short heating above the glass transition temperature (<1 s) of the crystals. It is unclear how this influences our compound II structures compared with the unaffected compound II, but some crystallographic data suggest that the influence on the Fe–O bond distance is minimal. Based on our crystallographic and spectroscopic data we suggest that for myoglobin the compound II intermediate consists of an Fe$^{IV}$–O$^*$ species with a single bond. The presence of Fe$^{IV}$ is indicated by a small isomer shift of $\delta = 0.07$ mm/s from Mössbauer spectroscopy. Earlier quantum refinements (crystallographic refinement where the molecular-mechanics potential is replaced by a quantum chemical calculation) and density functional theory calculations suggest that this intermediate H species is protonated.

Myoglobin (Mb)$^2$ is a heme protein found mainly in heart and skeletal muscle (1–3). Its function in oxygen storage and transportation has been well known for a long time, but more recently an understanding of Mb as a multifunction protein (functions as protection against oxidative damage and NO scavenging) has evolved (4–7).

The fact that Mb can react with hydrogen peroxide and give a ferryl species in a peroxidase-style manner (but with important differences, as will be detailed below) was studied already in the 1950s (8–10). For a classic peroxidase, like horseradish peroxidase (HRP), the reaction cycle involves a two-electron oxidation-reduction (Scheme 1). In the first classic HRP step the resting ferric (Fe$^{III}$) high spin (S = 5/2) form is oxidized by hydrogen peroxide to a water molecule and a heme state that is two oxidation equivalents higher (compound I) than the resting ferric form. This first step propagates through a hydroperoxy-intermediate (compound 0) where compound I is generated through a heterolytic cleavage of the O–O bond (11–13). The distal His is assumed to function as an acid/base catalyst to facilitate heterolytic cleavage by accepting a proton from the inner oxygen (oxygen ligated to the iron) and then donating it to the outer (leaving) oxygen. The negative charge on the leaving hydroxide during bond cleavage is then stabilized by the distal Arg in HRP (not found in Mb) (14, 15). Additionally, the proximal His can stabilize higher oxidation states on the heme iron by hydrogen bonding to a neighboring carboxylate group, thus making the Fe-bound nitrogen more negative (14, 15). The compound I intermediate formed is two oxidation equivalents higher than the resting form (Fe$^{III}$). One electron is withdrawn from the iron, resulting in a ferryl (Fe$^{IV}$ = O) state with a double-bonded oxygen atom and intermediate spin state (S = 1), which could interact magnetically with radicals. Depending on the peroxidase, the second oxidizing electron comes from either the porphyrin ring, giving a π-cation radical, or from an amino

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The atomic coordinates and structure factors (code 2V1E, 2V1F, 2V1G, 2V1H, 2V1I, 2V1J, 2V1K) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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$^2$ The abbreviations used are: Mb, myoglobin; HRP, horseradish peroxidase; P450, cytochrome P450; CPO, chloroperoxidase; CCP, cytochrome c peroxidase; Raman, resonance Raman; DFT, density functional theory; EXAFS, extended X-ray absorption fine structure; SNBL, Swiss-Norwegian Beam Line; ESRF, European Synchrotron Radiation Facility in Grenoble, France; oxyMb, oxymyoglobin; deoxyMb, deoxymyoglobin; MOPS, 4-morpholinepropanesulfonic acid; TAPS, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)]amino)-1-propanesulfonic acid.
Acid residue (Trp or Tyr) near the heme ring (16, 17). In the second step of the peroxidase reaction cycle compound I carries out a substrate (e.g. organic molecules A) oxidation (A → A⁺) resulting in a one-electron reduction to compound II (S = 1) by the loss of the heme/protein radical. The last step of the cycle is a further one-electron reduction of compound II to the resting ferric form accompanied again by a second substrate oxidation (A → A²⁺). Compound II was until a few years ago thought of as an Fe = O double bond state, but this changed when the crystal structures indicating a single-bond character were published (18, 19). The crystal structures of compound II all indicate this single-bond character, but some recent extended x-ray absorption fine structure (EXAFS) studies lean toward a double-bond character, so the question is still under some debate (20–22). Mb (Scheme 1 and Fig. 1) can react with hydrogen peroxide in a manner similar to peroxidases, including oxidizing several classes of small organic molecules, but at a much lower rate (23, 24). The reduced efficiency can be explained by the lack of three essential residues; the Asp that forms the hydrogen bond to the proximal His, the distal Arg that stabilizes the negative charge on the leaving group, and the distal His that is suggested to be too close to the heme to fully function as an acid-base catalyst during the heterolytic cleavage of the peroxide bond (14, 15, 25, 26). Through mutation studies Watanabe and co-workers (25, 27, 28) showed that mimicking the His distance in peroxidases increased the peroxidase activity of Mb and also led to the observation of compound I. Furthermore, a homolytic cleavage of the peroxide bond in compound 0 that gives Mb compound II and a hydroxyl radical have been suggested (29, 30). The Mb compound I has been observed in deuterated water (31). Formation of radicals was also observed in the reaction of Mb with hydrogen peroxide (32). For horse Mb the semi-stable radicals were shown to be a peroxyl radical on Trp-14 and a phenoxy radical at Tyr-103 (33).

The peroxidase intermediates described above are also found in heme-based oxygenases and catalases even though their reaction cycles are different. Several crystal structures of these intermediates have been solved (22). As mentioned above, active site amino acids of peroxidases are involved in deprotonation/protonation of the iron-linked peroxide in the first step of the reaction cycle. In contrast, it...
appears that for the monoxygenases cytochrome P450 (P450), nitric-oxide synthase, and heme oxygenases it is the solvent molecules that are responsible for the protonation of the dioxygen leading to the cleavage of the O–O bond (34). For P450 and nitric-oxide synthase as well as for bromo- and chloroperoxidase (CPO) the proximal ligand is not a His, but a Cys, and for most catalases it is a Tyr. These three residues differ in their electron donating capability to the iron, which can lead to some differences between these enzymes.

There have been some conflicting reports through the years regarding the two pH-dependent structural forms of compound II (35). For HRP two different distances for the Fe–O bond length in compound II (1.6 and 1.9 Å, respectively) were observed in EXAFS studies and suggested to be due the pH dependence of compound II (35–38). For Mb different spectroscopic products have been observed at high and low pH in the reaction with hydrogen peroxide (39–41).

The ferryl state of Mb is important, because it is believed to be of physiological relevance through its involvement in oxidative stress reactions (5, 7, 42). During ischemia oxymyoglobin (oxyMb) is converted to ferrous deoxymyoglobin (deoxyMb), which in turn is much more easily oxidized during reperfusion when reactive oxygen species are formed, initially to ferric Mb and then further to ferryl Mb (43, 44). This ferryl Mb can oxidize essential biological constituents (44), but it can be reduced back to ferric Mb if the supply of reductants is sufficient (43) and thereby plays a defensive role in protection of the heart by removal of reactive oxygen species (45). It has also been suggested that Mb can function as a detoxifier of both H₂O₂ and NO (46). High valent Mb may, however, have harmful effects by oxidizing lipids and be involved in muscle injury and subsequent renal failure, in which heme to protein cross-linked Mb and acidic pH play an important role (42, 47).

In the present study we try to elucidate the structure of Mb compound II by the use of crystallography in combination with spectroscopy and previous theoretical studies (22, 48). In the crystallographic studies the potential radiation damage and radiation-induced reduction of the metal-center must be addressed. The use of light absorption on small protein crystals, so-called single-crystal microspectrophotometry, is essential for generation of the correct oxidation state, and for monitoring changes of this state during data collection for proteins (19, 49, 50). About 90% of the interacting x-ray photons deposit their energy into the crystal lattice mainly through the photoelectric effect by generating electrons (50). This, together with further secondary electron emission, produces reactive species that can lead to radiation damage. The x-ray diffraction experiment then generates potential reducing equivalents (electrons) that can change the redox-state of metal-proteins (51). The importance of these questions and the use of single-crystal microspectrophotometry was acknowledged early in the study of these peroxidase, oxygenase, and catalase intermediates (52–54). The actual rate by which these intermediates are reduced varies considerably among proteins (22). The use of single-crystal microspectrophotometry is therefore essential to determine the redox-state correctly in each case. The use of crystallography in combination with microspectrophotometry, resonance Raman (rRaman), and Mössbauer spectroscopy suggests that compound II has a single Fe⁴⁺–O bond and is probably protonated.

**EXPERIMENTAL PROCEDURES**

**Purification and Crystallization**—Horse heart Mb (Sigma) was further purified by gel filtration on a Sephadex G75 column using the absorption ratio of 410/280 nm as the purification criterion. Mb was crystallized at three pH values: 5.2, 6.8, and 8.7. The crystals were grown at room temperature by batch methods with a 6–12 mg/ml Mb concentration and 80–85% of the crystallization stock solution (55, 56). The stock solution contained 3.9 m ammonium sulfate, 0.1 m buffer depending on the pH and 5–10% of glycerol. No buffer was applied at pH 5.2, whereas MOPS or HEPES was used at pH 6.8 and TAPS at pH 8.7. Rosette-shaped clusters of thin, plate-shaped crystals were grown within 1–7 days.

**X-ray Data Collection**—For each x-ray diffraction experiment a single crystal was separated from a rosette and transferred into a cryo-solution containing 75–80% crystallization stock solution and 20–25% glycerol. Compound II intermediates were prepared by 30 s of incubation in a cryo-solution to which had been added H₂O₂ to a final concentration of 22 mM, with subsequent flash freezing in liquid nitrogen. The diffraction data were mainly collected at the Swiss-Norwegian Beam Line (SNBL) BM01A at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, but also at beamline ID14-3 at ESRF and at beamline X11 at EMBL, DESY, Hamburg, Germany (Table 1). Usually, two separate scans, at high and low resolution, were collected using at SNBL a MAR345 image plate, at ID14-3 a ADSC Q4R charge-coupled device detector and at X11 a MARCCD 165-mm detector. Data collection temperatures were in the range of 100–110 K.

The diffraction data were processed with MOSFLM (57) and scaled with SCALA (58, 59) or processed with DENZO (60) and scaled with SCALEPACK (60) (Table 1). The first structure (PDB entry 1GIN (55)) was solved by molecular replacement with CNS (61) using PDB entry 1WLA as the starting model (62), whereas the 1GIN structure itself served as the starting model for the other structures (55). Refinements, including rigid body, simulated annealing, and conjugate gradient minimizations with CNS, and model improvements in O (63), were performed initially for some structures. Further steps included multiple cycles of restrained refinement in Refmac (58, 64) and model building in Coot (65) and addition of water molecules by ARP/wARP (66). In the later stages TLS refinement was introduced, and finally restrained anisotropic refinement performed with Refmac (67, 68). The anisotropy was monitored through the PARVATI program (69). Both steps lead to a significant drop in the Rcryst and Rfree values. All structures were finally refined with Refmac. No restraints were used for the Fe–N₇HEMS, Fe–N₇HIS, and Fe–O distances. The same hkl reflections were selected as Rfree set for all the structures. The figures were prepared with PyMOL.³ The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ.

³ W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
**TABLE 1**

Crystal data, data collection, and refinement statistics

| pH | Radiation-induced Mb comp. II | Radiation-induced ferric Mb | Radiation-induced Mb comp. II | Ferrous deoxy Mb | Radiation-induced Mb comp. II | Radiation-induced ferric Mb |
|----|-------------------------------|----------------------------|-------------------------------|----------------|-------------------------------|----------------------------|
| 5.2 | 5.2 | 6.8 | 6.8 | 6.8 | 8.7 | 8.7 |

**Crystal data**

- Space group: P2₁, P2₁, P2₁, P2₁, P2₁, P2₁, P2₁
- a (Å): 62.9, 63.2, 63.1, 62.9, 62.9, 63.0, 62.7
- b (Å): 28.7, 28.7, 28.7, 28.8, 28.8, 28.7, 28.5
- c (Å): 35.4, 35.6, 35.5, 35.4, 35.3, 35.6, 35.5
- β (°): 105.9, 105.8, 105.9, 105.7, 105.6, 105.7, 105.6

**Data collection**

- X-ray source: ESRF-BM01A, ESRF-BM01A, ESRF-BM01A, ESRF-BM01A, ESRF-ID14-3, ESRF-BM01A, EMBL-X11
- Wavelength (Å): 0.8730, 0.8727, 0.8000, 0.9000, 0.9312, 0.8727, 0.8496
- Temperature (K): 100, 110, 100, 110, 100, 110, 100
- Resolution range (Å): 20.8–1.35, 30.4–1.30, 26.8–1.30, 30.0–1.25, 22–1.20, 34.3–1.20, 21.9–1.40
- Completeness (%): 98.4/85.4, 98.2/98.8, 97.9/98.0, 97.3/95.8, 94.0/86.6, 99.1/99.1, 95.2/95.2
- Redundancy (%): 4.2/2.4, 2.5/2.3, 2.6/2.2, 2.6/2.3, 2.7/2.0, 2.5/2.3, 3.0/2.9
- I/σ(I): 22.0/2.7, 13.2/4.4, 13.5/2.5, 16.9/4.1, 16.4/3.4, 12.7/2.6, 24.2/2.8
- R cryst: 8.3/30.5, 4.2/18.3, 4.6/31.0, 3.5/18.0, 4.6/34.0, 4.4/35.5, 4.4/28.8

**Refinement statistics**

- R cryst (%): 13.9, 13.1, 14.8, 13.4, 13.7, 13.4, 12.9
- R free (%): 17.4, 16.5, 17.6, 16.4, 16.8, 16.3, 16.6
- Mean protein/solvent isotropic B (Å²): 11.3/22.4, 13.4/31.6, 12.4/30.1, 14.1/30.7, 10.6/27.0, 11.0/29.2, 13.3/28.7
- Ramachandran plot: ration in most favored/other allowed regions (%): 91.8/8.2, 92.5/7.5, 91.0/9.0, 91.8/8.2, 91.8/8.2, 91.8/8.2, 91.8/8.2
- Estimated overall coordinate error based on R cryst/maximum likelihood (%): 0.073/0.039, 0.056/0.030, 0.064/0.034, 0.050/0.029, 0.047/0.026, 0.044/0.029, 0.077/0.038
- Added waters: 190, 183, 182, 218, 214, 214, 201
- Volume not occupied by model (%): 3.9, 3.7, 3.7, 2.1, 2.1, 2.5, 3.5
- PDB code: 2V1G, 2V1H, 2V1E, 2V1K, 2V1I, 2V1F, 2V1I

Microspectrophotometry—The crystals described above were transferred between the microspectrophotometer and the diffractometer with cryo tongs (Hampton Research). Measurements were carried out on a microspectrophotometer system (4DX Systems AB, Uppsala, Sweden) on SNBL, whereas earlier measurements applied the Cryo-bench in connection with ID09 at ESRF (49).

EPR Measurements—As described previously (55), X-band EPR spectra were obtained on a Bruker ESP 300 equipped with an Oxford instrument 900 cryostat, and also at the Grenoble EPR (ID09 at ESRF (49)).

—57Fe-Mb was used for the measurements. The well documented acid/butanone method was used to remove the heme group of horse heart Mb (73–76). The apo-Mb was then reconstructed with heme enriched with 90% 57Fe. The 57Fe-reconstituted Mb behaved as native 56Fe Mb in a bath cryostat (Oxford MD 306) with a pair of permanent magnets. In all cases the spectra were analyzed by least-square fits using Lorentzian line shape.

The laser power at the sample point was adjusted to 8–10 miliwatts to prevent photoreduction and photodissociation. Raman shifts were calibrated with indene, CCl₄, and an aqueous solution of ferrocyanide. All measurements were performed with a spinning cell at room temperature 10–20 s after addition of hydrogen peroxide to samples consisting of 50–70 μM Mb. Samples of Mb crystals were frozen in crystallization mother liquor and kept at −30 °C or suspended in solution (an addition of 20% glycerol caused a substantial and unfortunate increase in fluorescence that prevented collection of useful Raman data with glycerol present). 90% 18O-enriched peroxide (IO 6325) was obtained from Icon Services (Summit, NJ) over 98% enriched D₂O and 18O-enriched water were from Cambridge Isotope laboratories, Inc (Andover, MA).

Mössbauer Measurements—The crystals described above were transferred between the microspectrophotometer and the diffractometer with cryo tongs (Hampton Research). Measurements were carried out on a microspectrophotometer system (4DX Systems AB, Uppsala, Sweden) on SNBL, whereas earlier measurements applied the Cryo-bench in connection with ID09 at ESRF (49).

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RESULTS

Formation of Mb Compound II in Crystals

Light Absorption Spectra of Ferric Mb and Compound II—Formation of the Mb compound II intermediate from resting high spin ($S = 5/2$) ferric Mb can be monitored by light absorption of the heme group, which is sensitive to the oxidation and spin states of the heme iron. Spectra for crystalline samples and for solutions will have some different features as will spectra recorded at different temperatures. The sensitive microspectrophotometer is easily saturated by strongly absorbing proteins and especially in the crystalline state, which is highly concentrated (49, 50). Measurements are therefore possible only on relatively thin crystals, and the highly absorbing Soret peaks are not determined accurately. The spectrum of a crystal depends on the orientation in the incident light beam due to its anisotropy (except for cubic systems) (50). For our plate-shaped Mb crystals we can only observe a minimal change in the peaks upon rotation, because the spectrophotometer is easily saturated when the thicker side blocks the light path. In Fig. 2 the spectra of both ferric Mb and Mb compound II at pH 6.8 (obtained after incubation with $H_2O_2$ at room temperature) are shown for a solution at room temperature, for a crystal at room temperature, for a frozen solution at 110 K, and for a crystal at 110 K all in cryo-solution. The resting ferric form (also called met-form) gives rise to relatively wide visible peaks at 500 and 638 nm both in solution (room temperature) and as a frozen glass (110 K), but at 110 K one can observe a tendency for splitting of the latter peak. In the crystalline state two additional peaks at 542 and 580 nm can be observed at both room temperature and at 110 K, with a distinct splitting of the 638 nm peak at 110 K. The spectra observed at pH 5.2 are very similar to those obtained at pH 6.8, but at pH 8.7 (low spin) only the peaks at 542 and 580 nm are observed. The solution spectrum of Mb compound II at room temperature has two wide peaks at 546 and 583 nm, which are slightly sharper for the crystal at room temperature, but become clearly sharper at 110 K with the additional appearance of a shoulder at 596 nm and slightly shifted peak positions (542, 580, and 596 nm). The additional peak at 596 nm is the feature that distinguishes Mb compound II from oxyMb or Mb compound III (both these species are resonance forms of ferrous-oxygen complex and ferric-superoxide complex). In going from ferric Mb to Mb compound II there is also a shift in the Soret peak, observed both in solution and for a thin crystal, from 410 to 422 nm (data not shown in Fig. 2).

EPR Spectroscopy of Compound II in Crystals—The reaction of Mb crystals with hydrogen peroxide was initially monitored by EPR spectroscopy. To obtain a detectable EPR signal of the heme-iron more than a hundred small Mb crystals (in the standard cryo-solution used for x-ray diffraction experiments) were used in each experiment. After 30 s of incubation with 22 mM hydrogen peroxide at either pH 6.8 or pH 5.2 (55) the resting high spin ferric Mb ($S = 5/2$) X-band EPR signal (from lowest Kramer doublets) with $g$ values at 5.9, 5.9, and 2.0 was virtually gone (<2% remain). At pH 8.7 resting ferric Mb has, in addition to high spin, a large portion of ferric low spin ($S = 1/2$). Both the high and low spin signals disappear after 30 s of incubation with 22 mM hydrogen peroxide. This observation, together with the lack of observation of any other iron EPR signal (in the temperature range 3.6–77 K), indicate that the species formed has integer spin. Additionally, small amounts (0.2–2%) of two radical signals were detected. A typical peroxyl-Trp radical signal with no hyperfine coupling ($g$ values 2.036, 2.006, and 2.006) (77, 78) was observed after 30 s of hydrogen peroxide incubation at pH 5.2 and at pH 6.8, whereas a 16 G broad non-peroxyl signal ($g$ value 2.005) was observed after 300 s incubation at pH 5.2 and 6.8 as well as after 30 or 300 s at pH 8.7. The presence of small amounts of radicals is in agreement with reported catalase activity of our Mb crystals (55) and with a very low steady-state amount of compound I before formation of compound II. The observation of a low radical content in combination with the absence of an iron EPR signal is consistent with the presence of compound II in the crystals after hydrogen peroxide treatment. The above experiments also demonstrate that the Mb compound II state is stable for at least 5 min in crystals at room temperature. Attempts were made to study the integer spin ($S = 1$) EPR signals with high field EPR in the frequency range 95–285 GHz at 4–25 K, but the signals from crystals were too weak or broad to be observed at the current protein concentrations. This type of single-pass transmission EPR spectrometer without cavity is not sensitive for broad signals from proteins, whereas sharper integer spin EPR signals from small inorganic complexes and/or solids can be observed (71, 72).

Resonance Raman Spectroscopy of Mb Compound II in Crystals—The Raman probe to analyze the oxidation/spin state and the binding of ligands to the heme iron (79–84). Fig. 3 shows low resolution (~2 cm$^{-1}$ resolved) Soret excitation at 413 nm rRaman spectra between 600 and 1600 cm$^{-1}$ of resting ferric (lower trace) and hydrogen peroxide treated Mb (middle trace) at pH 6.8. The spectra show typical Raman shifts in accordance with full conversion of ferric Mb to Mb compound
II (85, 86) as seen by the shift of the oxidation state $\nu_4$ heme core mode from 1369–1370 to 1375 cm$^{-1}$ and by the decrease of the ferric $\nu_2$ heme core mode at 1563 cm$^{-1}$. Similar shifts (Fig. 3) were observed for several Mb crystals measured in the crystallization solution (or as frozen crystals at $-30^\circ C$), and a shift of pH between 5.2, 6.8 and 8.7 also did not change the rRaman spectra (data not shown). The presence or absence of 3 M ammonium sulfate (which has a strong symmetric sulfate mode at 983 cm$^{-1}$) or presence of crystalline state did not seem to influence the characteristics of the heme Raman spectra. Mb rRaman experiments with $>95\%$ exchange with D$_2$O did not change the low resolution spectra significantly; only insignificant shifts ($<1$ wavenumber) were observed with variable experimental conditions. The rRaman experiments as well as light absorption and EPR spectra (and in agreement with Mössbauer spectroscopy below) show that Mb in crystals indeed exhibit formation of compound II.

**Light Absorption Spectra of the Mb Compound II Crystals**—Because metal centers in heme proteins can be reduced after x-ray exposure, the Mb compound II crystals were controlled by microspectrophotometry after data collection. In Fig. 4 the light absorption spectrum of the pH 8.7 Mb compound II crystal after data collection (normal dataset, 4–6 h of exposure at SNBL (BM01A, ESRF)) is compared with the spectra of the same non-exposed compound II crystal and to that of a ferric Mb crystal. A change in the compound II spectrum is manifested by the appearance of a peak at 567 nm as the 580 and 595 nm peaks decay during the crystallographic data collection. Similar changes were observed at pH 5.2 and 6.8. These changes did not result in a normal ferric ($S = 5/2$) or ferrous ($S = 2$) Mb state. More rapid Mb compound II datasets were collected with 1/10 of the exposure time of a normal dataset at SNBL (rapid dataset, 45 min of exposure at SNBL).

![FIGURE 3. Low resolution resonance Raman spectra of Mb compound II, in solution and Mb-crystals. a, lower trace, resting ferric Mb (70 $\mu$m at pH 6.8) with $\lambda_{ex} = 413$ nm (resolution 2 cm$^{-1}$, 1800 grating; middle trace, after 30 s incubation of with 0.1 mM of H$_2$O$_2$; higher trace, difference spectra between lower and middle traces. b, lower trace, resting ferric Mb crystals at pH 6.8 with $\lambda_{ex} = 413$ nm (resolution 2 cm$^{-1}$); middle trace, after 30-s incubation of with 2.2 mM H$_2$O$_2$; higher trace, difference spectra between lower and middle traces (8 milliwatts at protein solution or protein crystal).]
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FIGURE 4. Single-crystal light absorption spectra of the Mb compound II, pH 8.7, crystal before and after data collection of a normal dataset (4–6 h of exposure at SNBL (BM01A, ESRF)) and a rapid dataset (45 min) at both pH 5.2 and 6.8, including spectra after annealing. Additionally, one of the first spectra recorded after data collection of compound II is shown (probably after annealing). The resting ferric Mb spectrum is shown at pH 5.2, 6.8, and 8.7 as a reference.

(BM01A, ESRF), which showed similar features (Fig. 4). Because the radiation-induced changes have not progressed very far, these states contain a combination of the normal compound II and the radiation-induced compound II state (Fig. 4, only pH 5.2 and 8.7 shown, but similar at pH 6.8). These radiation-influenced compound II states are immediately regenerated to the normal compound II state if the cryo-stream was blocked for 1 s or less (Fig. 4) with only a very small amount of ferric Mb. A rapid blocking of the cryo-stream (110 K), so-called annealing, is a well known crystallographic technique for removing ice on the crystal.

Crystal Structure of Radiation-induced Mb Compound II—In Fig. 5 the crystal structures of the heme region in Mb compound II at pH 5.2 (55), 6.8, and 8.7 are shown with the electron density $2F_o - F_c$ map, the final $F_o - F_c$ map, and $F_o - F_c$ maps with the iron-ligated oxygen atom omitted for map calculation. The difference electron density map nicely defines the oxygen atom, and refinements indicate full occupancy for the oxygen atom. The iron-ligand distances (including the Fe–O distances) were unrestrained during the refinement. For quite a few of the residues alternative conformations have been introduced and for the pH 8.7 structures also for one of the propionates in the heme group. In Fig. 6 the heme region of the radiation-induced Mb compound II crystal structures are compared with each other at the three different pH values and to the radiation-induced ferric Mb structures. The Fe–O distances, the Fe to proximal His distances, the Fe to distal His distances, and the hydrogen bond distances between the oxygen and the distal His are listed in Fig. 6. Crystal structures of Mb compound II show a relatively long Fe–O bond of 1.86–1.90 Å in the full pH range studied (5.2–8.7). This bond distance is ~0.3 Å longer than expected for an oxoferryl (Fe$^{IV}$=O) species and ~0.2 Å shorter than in the radiation-induced ferric Mb structures as well as in other reported ferric Mb structures (87). The compound II structures solved from the crystals with the shortest exposure times (giving a lower resolution of 1.5 Å, data not shown) show the same Fe–O bond lengths of 1.86–1.90 Å. There was no correlation between the different Fe–O lengths and the pH for compound II, because we had structures at pH 5.2 with Fe–O distance of 1.86 Å, and at pH 8.7 with 1.90 Å (data not shown). The 0.05-Å difference was either the actual uncertainty or an indication of trapping of different isoelectric forms of Mb compound II (see "Discussion"). A possible pH-induced effect was the elongation (~0.1 Å from pH 5.2 to 8.7) of the iron-ligated oxygen to distal His distance, which was probably due to some deprotonation. Compound II and ferric Mb showed no other significant differences in the heme region. The small changes described above were distinguishable due to the high resolution of these crystal structures. The estimated overall coordinate error from Refmac was 0.044 Å or 0.029 Å for the 1.20-Å resolution compound II structure based upon the $R$ value or maximum likelihood, respectively. This is smaller than the observed differences between a single and a double bond but in the same order or larger than the 0.05 Å Fe–O shift between different Mb compound II structures. Additionally, the different states have been treated in the same way throughout the refinement (without restraints), and the positions of the iron-ligated oxygen atom has also been confirmed by omit maps. In the original published 1GJN structure three water molecules were modeled in close to the distal His. After re-refinement with introduction of anisotropy the difference electron density map revealed obvious additional density between the three water molecules (Fig. 1) at what would seem to be the carbon positions in glycerol. The glycerol molecule is hydrogen-bonded to the ND1 of the distal His (Fig. 1) and is located at all three pH values for the radiation-induced compound II as well as for the radiation-induced resting state. Fig. 1 also shows the final pH 5.2 compound II electron density maps ($2F_o - F_c$) of the Trp-14 and Tyr-103 with the electron difference map. Similar observations devoid of significant additional positive or negative density were also made for compound II at pH 6.8 and 8.7. The electron difference density maps show some negative density at several carboxylate groups (map/structure not shown), which indicate that some general radiation damage had occurred.

When Mb crystals were exposed to 22 mM H$_2$O$_2$, compound II was generated within seconds, and it remained quite stable in this environment. Structures for crystals that were incubated for 5 min in 22 mM H$_2$O$_2$ are indistinguishable from those obtain with only 30 s of incubation, and no heme to protein linkages have been observed for the Mb compound II crystals as reported for Mb solutions (88). After incubations longer than 15 min the crystals start to lose their color indicating breakdown of the heme group.
FIGURE 5. Crystal structures of the heme region. Structures for radiation-induced Mb compound II (resolutions of 1.35, 1.30, and 1.20 Å, respectively) at pH 5.2, 6.8, and 8.7, radiation-induced ferric Mb (resolutions of 1.30, 1.20, and 1.40 Å, respectively) at pH 5.2, 6.8, and 8.7, and ferrous deoxyMb (resolution of 1.25 Å) are shown with the electron density $2F_O - F_C$ map (contoured at 1σ in gold), the final $F_O - F_C$ map (contoured at +3σ in green and at −3σ in red), and electron density difference $F_O - F_C$ maps with the iron-ligated oxygen atom (or water above the heme ring in deoxyMb) omitted for map calculation (contoured at 5σ in magenta for the iron-ligated oxygen atom and at 4.5σ for the water in deoxyMb).
Mo¨ssbauer Spectroscopy of Mb Compound II—Mo¨ssbauer spectroscopy is a convenient tool for detecting all Fe states, as well as those that are EPR-silent (89–95). As mentioned above, we could not observe any integer spin EPR or high field (285 GHz) EPR signals for an $S/H_2O$ state. Earlier magnetic Mo¨ssbauer studies and low temperature magnetic CD studies of Mb compound II have shown that Mb compound II is in an $S/H_2O$ state (40, 96, 97). The magnetic CD studies indicated the presence of 5% FeIII at low pH or the presence of a different $S/H_2O$ state with negative $D$ value (40). We examined Mb compound II in the pH range 5.2–6.8 in the presence of glycerol and sulfate as used in the crystallographic experiments. The Mo¨ssbauer spectra in Fig. 7 of Mb compound II solution at pH 5 had $Fe (mm/s) = 0.07$ and $E_Q (mm/s) = 1.46$, which indicate that $95\%$ is in an FeIV state (Table 2). At pH 7, the largest component (89%) exhibited similar FeIV Mo¨ssbauer parameters with $Fe (mm/s) = 0.07$ and $E_Q (mm/s) = 1.48$. The second small component with $Fe (mm/s) = 0.42$ and $E_Q (mm/s) = 1.96$ was assigned to ferric heme iron not oxidized by $H_2O_2$. These $Fe$ and $E_Q$ values are in agreement with previous studies of Mb, $Fe = 0.09–0.07$ mm/s and $E_Q = 1.54–1.48$ mm/s (96, 97).

TABLE 2
Oxidized Mb at pH 6.8 and 5.2 Mo¨ssbauer parameters
Recorded at 77 K and in a 20-millitesla perpendicular field.

| pH   | FeA | FeB | Fe |
|------|-----|-----|----|
| 6.8  | 0.07| 0.33| 1.48|
| 5.2  | 0.42| 0.33| 1.46|

Mössbauer Spectroscopy of Mb Compound II—Mo¨ssbauer spectroscopy is a convenient tool for detecting all Fe states, as well as those that are EPR-silent (89–95). As mentioned above, we could not observe any integer spin EPR or high field (285 GHz) EPR signals for an $S = 1$ state. Earlier magnetic Mo¨ssbauer studies and low temperature magnetic CD studies of Mb compound II have shown that Mb compound II is in an $S = 1$ state (40, 96, 97). The magnetic CD studies indicated the presence of 5% FeIII at low pH or the presence of a different $S = 1$ state with negative $D$ value (40). We examined Mb compound II in the pH range 5.2–6.8 in the presence of glycerol and sulfate as used in the crystallographic experiments. The Mo¨ssbauer spectra in Fig. 7 of Mb compound II solution at pH 5 had $Fe (mm/s) = 0.07$ mm/s and $E_Q (mm/s) = 1.46$ mm/s, which indicate that >95% is in an FeIV state (Table 2). At pH 7, the largest component (89%) exhibited similar FeIV Mo¨ssbauer parameters with $Fe (mm/s) = 0.07$ mm/s and $E_Q (mm/s) = 1.48$ mm/s. The second small component with $Fe (mm/s) = 0.42$ mm/s and $E_Q (mm/s) = 1.96$ mm/s was assigned to ferric heme iron not oxidized by $H_2O_2$. These $Fe$ and $E_Q$ values are in agreement with previous studies of Mb, $Fe = 0.09–0.07$ mm/s and $E_Q = 1.54–1.48$ mm/s (96, 97).

Radiation-induced Ferric Mb and DeoxyMb

Light Absorption Spectra of Ferric Mb—The light absorption studies of Mb crystals show that the ferric state was not stable

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**FIGURE 6.** Crystal structures of the heme region of the radiation-induced Mb compound II crystal structure at pH 5.2 (red), 6.8 (green), and 8.7 (blue) compared with each other and to the radiation-induced ferric Mb structures (pale colors). The Fe–O distances, the iron to proximal His distances, the iron to distal His distances, and the hydrogen bond distances between the oxygen atom and the distal His are shown.

**FIGURE 7.** Mössbauer spectra of Mb compound II at pH 5.2 and 6.8 both show the presence of FeIV. **a**. 1 mm$^{257}$Fe Mb at pH 5.2 after incubation with 22 mm hydrogen peroxide for 30 s, then frozen, and the spectrum measured at 77 K yielding $Fe (mm/s) = 0.07$ and $E_Q (mm/s) = 1.46$. **b**. Same as in **a** but at pH 6.8. Subspectrum A (FeA) represents 89% FeIV, $Fe (mm/s) = 0.07$, and $E_Q (mm/s) = 1.48$; and FeB represents 11% FeII, $Fe (mm/s) = 0.42$, and $E_Q (mm/s) = 1.96$. Sim represents the envelope of FeA and FeB.
during crystallographic data collection. In Fig. 8 the light absorption spectra after a normal high resolution data collection are shown at pH 6.8 (normal dataset). The normal ferric Mb peaks at 500, 540, 580, and 635 nm disappeared, while new peaks were formed at 525 and 565 nm. Two additional new peaks at 535 and 555 nm could be observed at different crystal orientations or in different crystals, which could indicate two different states of the radiation-induced ferric Mb. The Mb crystals undergo some type of radiation damage, but as seen from Fig. 8 ferric Mb was not reduced to high spin \( S = 2 \) ferrous deoxyMb (five-coordinated Fe\(^{II} \) with the iron atom below the heme plane). The radiation damage could be inducing a low spin \( S = 0 \) ferrous species with water or hydroxide bound to the iron atom. To try to reduce the radiation damage a rapid data collection of \( \frac{1}{20} \) of the normal x-ray exposure time (in total 15 min at SNBL/BM01A at ESRF) was performed. The light absorption spectra showed a mixture of the normal ferric Mb and the radiation-induced ferric Mb (Fig. 8, rapid data set). Using the previously described annealing technique at the radiation-induced ferric Mb crystals, the normal ferric Mb spectrum was regenerated (Fig. 8).

**Crystal Structure of Resting Ferric/Ferrous Mb**—The crystal structures of the radiation-induced ferric Mb are shown in Fig. 6 at pH 5.2, 6.8, and 8.7. These three structures are similar with an Fe–O distance of 2.0 Å. Both the radiation-induced ferric Mb and compound II show quite similar structures with the main difference being the 0.2-Å shift in the Fe–O distance. The ferrous deoxyMb structure (spectrum shown in Fig. 8) shows the well known five-coordinated structure with the iron atom slightly below the heme plane and a water molecule is found 3.0 Å above the NC-pyrrole nitrogen atom in the heme ring. Data for the ferric Mb crystal that was only exposed for 15 min at SNBL (rapid data set, Fig. 8) yielded a 0.1- to 0.2-Å longer Fe–O distance than the pure radiation-induced ferric Mb structures.

**DISCUSSION**

The focus on the possible reduction of the metal center in heme proteins by synchrotron radiation has, as mentioned above, become more evident from the studies of HRP (19). We have previously stated that we observe radiation-induced changes to ferric Mb, but not to Mb compound II, and this was our interpretation until recently (22). At the time of this study only an off-line microspectrophotometer was available at BM01 (ESRF), where we carried out our main experiments. The required transfer of the crystal between the diffractometer and the microspectrophotometer can potentially give rise to small heating when not carried out fast and carefully enough. During removal of the crystal with cryotongs, it takes only 1–2 s for the crystal to reach the glass transition temperature (98). Heating of a radiation-damaged crystal will lead to increased movements of the radicals and consequently to damage and destruction of the crystal. We therefore expected that a small heating of the Mb compound II crystals after data collection would either reduce (keep reduced) or destroy the crystal. The spectra showed only the normal compound II features after data collection and transfer of the crystal to the microspectrophotometer (Fig. 4, first dataset). Further investigations have shown that a small heating (annealing) of these Mb compound II crystals results in a regeneration of compound II state (Fig. 4), and that Mb compound II does experience radiation-induced changes during data collection (Fig. 4). We would have expected a reduction to a stable ferric or ferrous state as observed for HRP and not to a semi-stable state (intermediate H, see below). This shows a clear difference between Mb and HRP in the ability to become reduced during data collection and further complicates the picture of potentially radiation-damaged metal sites. For HRP both compound II and the ferric state are reduced to stable states, whereas for cytochrome c peroxidase (CCP) neither appears to be reduced (19, 22, 99). However, the results for CCP can be questioned after the observation in Mb that the original state is regenerated during heating, because the CCP crystal was dissolved before light absorption spectra were recorded. This underscores the difficulty of predicting the correct state without the use of single-crystal microspectrophotometry. The heme environment must be a fine-tuned system, where the propensity to be reduced through radiation damage is affected by small modifications. Further investigation into these differences must be made.

**Compound II and Intermediate H**—The Mb compound II crystal structures solved show, as discussed above, a radiation-induced semi-stable state that will be referred to as intermediate H (Scheme 1) due to it being protonated. It is not clear how this radiation-induced compound II state is modified in comparison to the true compound II state. We have solved crystal structures of compound II with a reduced total exposure time (1/20 and 1/10 of a normal dataset giving consequently a lower resolution of 1.5 Å) at all the three pH values (data not shown). The single-crystal light absorption spectra in Fig. 6 show that these structures have only partially changed to the radiation-induced states. The structure of the reaction site is essentially
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the same as that observed for the fully radiation-induced ones. The Fe–O distances are between 1.86 and 1.90 Å, which indicates that it is not prone to change during the spectroscopically radiation-induced changes. For the ferric state, however, we observed a lengthening of the Fe–O distance upon reduced radiation exposure time (see discussion below). We still believe that compound II in Mb has a single bond of 1.86–1.90 Å.

Our goal has been to bring deeper insight into the structure of the intermediates in the peroxidase reaction cycle. Compounds I and II have been studied for quite some years. The publication of two EXAFS studies of HRP compound II giving two different results with respect to the Fe–O distance, both short (1.64 Å) and long (1.93 Å), has led to some debate regarding the state of compound II (36, 37). The view of the Fe–O as a double bond had gained much support until the publication of compound II in Mb and HRP and compound I in CCP, which showed a single bond. The discussion is still on-going, and EXAFS and crystallography in particular tend to give contradictory results. For HRP an unreduced compound II state has fully been solved by crystallography, but the Fe–O distance is still longer than that estimated from equivalent EXAFS studies (19, 21).

Green and co-workers have in the recent years made both theoretical calculations and EXAFS studies that support their hypotheses that the short/long Fe–O distance differences arise from the property of the proximal ligand. They argue that compound II with proximal Cys donates more electrons resulting in a long Fe–O bond for P450 and CPO compound II, whereas His does not contribute in the same way resulting in a short bond for HRP, CCP, and Mb (21, 100–102). The recent crystal structures of Mb, HRP, CCP, and catalase show, on the other hand, quite uniformly a compound II state (or compound I with protein radicals) with a relatively long Fe–O bond. This is now further supported for Mb in the pH range 5.2–8.7. Nevertheless, all the EXAFS studies (with the one exception) show a short Fe–O bond for His ligated compound II. A concern raised has been that the crystal structures might be reduced by the intense synchrotron radiation resulting in a longer Fe–O bond. For HRP and Mb this has been monitored by single-crystal light absorption. Reduction was observed for HRP but overcome by collecting unreduced fractional datasets. As mentioned above Mb compound II experiences radiation-induced spectroscopic changes, but it does not seem to influence the Fe–O distance. The difference between EXAFS and x-ray crystallography studies might arise from differences between Mb compound II in solution and in crystals as well as differences in the solvent solutions. However, we did not observe a difference between solution and crystal in the rRaman experiment, and the relatively long Fe–O bond persisted in solution.

The FeIII–OH stretching modes are assigned in the 490–570 cm⁻¹ region, while FeIV=O modes have been assigned at 797 cm⁻¹ for Mb compound II (and recently also at 790 at low and 804 cm⁻¹ at high pH), 779 cm⁻¹ for HRP compound II, and 767 cm⁻¹ (753 cm⁻¹) for CCP compound I (85, 86, 100, 103–105). These frequencies are downshifted compared with synthetic model compounds probably due to hydrogen bonding to the ferryl group by residues in the heme pocket (105). We have some experiments indicating the possibility of another 18O-peroxide-sensitive mode at around 690 cm⁻¹, which would lie between the FeIII–OH and FeIV=O stretching modes in energy and could be in agreement with a calculated but unscaled FeIV=O–OH mode (101). However, due to smaller than expected 18O-shift, lack of deuterium shift, and the possibility of a mixture with the previously accepted ferryl mode, further investigations are needed. The previous DFT calculations and quantum refinement based upon our pH 5.2 data set of intermediate H clearly show the presence of a proton (48). An alternative explanation of the above observations might be that the intermediate H observed in the crystal structures is protonated, whereas the normal compound II observed in rRaman and by Mössbauer spectroscopy might be unprotonated. Until recently no Fe–O rRaman mode for CPO compound II had been observed, and it was presumed to be due to ferryl oxygen exchange with water (105) implying that CPO is different from HRP, CCP, and Mb with an FeIV=O–OH state for compound II (21). This has been supported by the recent observation of a 18O- and D-sensitive mode as low as 565 cm⁻¹ in CPO, which could indicate an FeIV=O–OH (106). However, this places the Fe–O mode in the area of FeIII–OH stretching modes (490–570 cm⁻¹), which seems a little low in energy.

The differences in the HRP compound II lengths in EXAFS of 1.64 and 1.93 Å have been suggested to arise from the known pH dependence of HRP compound II (35, 105). Chang et al. (38) showed by EXAFS that at pH 7 the Fe–O distance was 1.90 and decreased to 1.72 at pH 10. The rrRaman frequencies also change with pH, but the shifts were smaller than expected from the EXAFS bond distances by the use of Badger’s rule (38, 86, 107, 108). However, similar results have been reported for synthetic models (38, 109). The pH dependence in HRP is believed to originate from the protonation state of the distal His–42 and a reorientation of the water, which hydrogen bonds to the ferryl oxygen (105). Recent Mössbauer studies on catalase compound II and EXAFS studies on CPO compound II have shown pH-dependent changes in these proteins as well (102, 110). Several studies have proposed a pH dependence of compound II in Mb (39–41, 111), and a low and high pH form with different absorption spectra is observed at pH 6 and 8.6, respectively (41). However, for Mb the 797 cm⁻¹ oxoferryl rRaman mode is insensitive to pH changes in the range from 6 to 12 (112, 113). From our crystallographic Mb compound II studies we were not able to detect any change in the Fe–O distance in the pH range 5.2–8.7 nor any differences in single-crystal light absorption spectra (Figs. 4 and 6).

Newer Mössbauer and DFT studies on CPO, P450, and catalase show that a protonated ferryl species FeIV–OH would have an enlargement in the quadrupole splitting parameters compared with an unprotonated ferryl FeIV–O (110, 114, 115). From Mössbauer experiments the ΔE_Q for protonated ferryls are observed to vary between 2.06 and 2.29 mm/s for CPO, P450, and catalase, whereas for unprotonated ferryl it is observed to be 1.59 and 1.47 mm/s for CPO and catalase (110, 114, 115). DFT calculations on Mb indicate that the ΔE_Q for unprotonated and protonated ferryl Mb should be 1.00 and 2.75, respectively (100). These results could then indicate that in solution our Mb compound II (ΔE_Q = 1.48 mm/s) should consist of an unprotonated ferryl state, whereas intermediate H
in crystals still is a protonated ferryl state as shown by quantum refinement (48). However, as mentioned above we did not observe a shortening of the Fe–O bond in the partially radiation-induced data sets, which indicates that compound II should also have a relatively long Fe–O bond.

Quite a few synthetic high valent heme model complexes have been prepared (105). The rRaman stretching frequencies for the synthetic heme FeIV=O complexes are generally found at higher energies (861–763 cm⁻¹) than in the activated protein system (815-753 cm⁻¹), but the variation is large among the different complexes (105). There are no FeIV–OH complexes listed in the review by Terner et al. (105), but an FeIV–OH at 690 cm⁻¹ would position itself between the oxoferryl and the FeIII–OH (553-491 cm⁻¹) frequencies. Relevant heme model complexes for comparison with a compound II structure are missing, because no crystal structures of heme FeIV–O/OH complexes are available in the Cambridge Structural Data base (Version 5.27 of May, 2006) (29), which contains only a couple of FeIV heme complexes with relatively long Fe–O bonds (2.078–2.159) and with the heme iron linked to another heme iron or manganese by a μ–N bridge (116, 117). For non-heme FeIV a few synthetic model complexes have been synthesized (118), and two of the non-heme FeIV=O complexes have been solved by x-ray crystallography giving Fe–O distances of 1.646 (3) and 1.639 (5) (118–120). Additionally, one synthetic non-heme FeIV complex with an alkylperoxo and a hydroxide ligand show from EXAFS one or two Fe–O/N bond(s) of 1.82 Å and two rRaman modes at around 678 and 685 cm⁻¹ (118, 121). The non-heme oxoferryl complexes confirm that such a species has an Fe–O double bond but not how it bonds in a protein. However, one of the non-heme FeIV complexes indicates that a structure with an Fe–O distance of ~1.8 Å would have a rRaman Fe–O mode in the area of ~680 cm⁻¹.

The compound I and compound II states in heme proteins have been extensively studied during the last years with theoretical methods by a number of different research groups (48, 122–126). Several have shown that a protonated compound II state results in a Fe–O bond distances in accordance with the recent crystal structures and proposed that the active compound II state is the protonated form (21, 48, 124, 126–128). It is still under debate whether the presence of a protonated form is general or true only for the thiolate systems with the higher proton affinity compared with His-ligated systems (22, 100, 126). Our radiation-induced Mb compound II crystal structures as well as the HRP, CCP, and catalase structures show, however, a state with a long Fe–O bond in accordance with a protonated state.

By previous calculations the Fe–O bond length of the protonated compound II state is shown to be considerably affected by the protonation state of the distal His (22, 48). The distal His can be a hydrogen bond acceptor or donor (with one or both nitrogens protonated). DFT calculations have shown that the Fe–O distance will increase by 0.1 Å when the FeIV–OH goes from being a donor to becoming an acceptor of a hydrogen bond to the distal His (48). Derat and Shaik (129) recently presented results for different isoelectric forms of compound II: FeIV=O, 1.72 Å; FeIV–OH, 1.80 Å; FeIII–OH₂⁺, 1.87 Å (porphyrin radical); and FeIII–OH₂⁺, 1.92 Å (porphyrin radical). They propose that trapping these different isoforms of compound II can explain the differences observed in HRP by EXAFS (129). The different forms could be relevant for the characterization of a putative intermediate H. The FeIII–OH₂⁺ shows a bond that is in accordance with our crystal structure of Mb at pH 5.2, whereas the FeIV–OH is in accordance with our structure at pH 8.7. A protonated ferric hydroxyl–radical would be in accordance with the magnetic CD results showing a small portion of FeIII at pH 3.5 but not with our Mössbauer studies at pH 5.2 (40). If these states are interconverted fairly easily, different methods could result in trapping of different forms. The extra energy from x-ray radiation might increase the amount of protonated forms. Whether these isoelectric forms could explain the differences observed for the Fe–O distance in Mb (1.86 versus 1.90 Å) is difficult to say because a 0.05-Å difference is at the border of the uncertainty of the crystallographic structures. A pH effect from a protonation/deprotonation of the distal His in Mb is less likely, because structures with both 1.86 and 1.90 Å were observed at all the three pH values studied.

**Resting State** — In the 1970s Gasyna (130) showed that ferric Mb can be one-electron reduced by radioisotopes of ⁶⁰Co at 77 K. This is the same effect that we observed for our ferric Mb crystals and that Engler et al. (131) observed after x-ray exposure. A reduction at low temperature 77–110 K probably reduces the FeIII to FeII, but at this temperature the water that ligates to the iron will not be able to move away to generate the normal ferrous deoxy state (132, 133). The intermediate state is most probably a low spin (S = 0) ferrous FeIII–H₂O or FeIII–OH state. Our single-crystal light absorption spectra of this low spin ferrous state are similar to the ones described previously for frozen solutions (130, 132). A very recent and thorough survey on cryoradiolytic reduction of crystalline heme proteins has demonstrated a very fast reduction of ferric Mb (134), which confirms our observations. In frozen solutions the low spin ferrous intermediates relax to the normal high spin ferrous deoxy Mb during heating (132, 133). When we carried out a short annealing of this state in crystals it was the high spin ferric Mb state that was regenerated and not the ferrous deoxy state. The generated state must be very unstable at higher temperatures in the crystal, and the water molecule must be unable to escape, so a regeneration of the ferric state is more favorable than the ferrous deoxy state. This marks a difference between the cryoradiolytic reduction in solutions and crystals and might point toward a strategy for collecting unreduced ferric Mb structures.

The Fe–O bond length in the partially radiation-induced ferric structure was 0.1 Å longer than in the completely reduced structure. This dataset has lower quality and resolution than the fully reduced datasets, resulting in higher uncertainty in the determination of the Fe–O distance, but our observations are supported by Engler et al. (131) who also observed a shortening of the Fe–O distance upon reduction. Why is the Fe–O distance shorter in the FeIII-ligated than in the FeIII-ligated structure when the charge is reduced? We believe that this is an indication of an FeIII–OH state instead of an FeIII–OH₂ for the FeIII-ligated Mb structure. It can be noted that for ferric Mb the EXAFS studies result in shorter Fe–O distances than the x-ray diffraction results: 1.88 Å and 2.2 Å, respectively (70).
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CONCLUSION

We have previously reported a long Fe–O bond for Mb compound II (55). Further crystallographic investigations in the pH range 5.2–8.7 and previously reported quantum refinements support an Fe–O single bond where the oxygen is protonated FeV–OH of intermediate H. The compound II state in the Mb crystal structures has been investigated by the use of single-crystal light absorption, and radiation-induced changes were observed resulting in an unknown unstable intermediate H, which by heating regenerates compound II. The intermediate H is not a known stable ferric or ferrous state. Some structural observations indicate that there is no significant difference in the Fe–O distance between intermediate H and compound II. We therefore believe that intermediate H is an FeV–OH state (or even FeIII–OH(2) and porphyrin radical) with compound II being similar but possibly unprotonated. Since the publication of the Mb compound II structure, a long Fe–O bond for compound II has been further supported by crystallographic studies on HRP, catalases, and the isoelectric compound I in CCP. This is probably a general feature of the compound II states and the isoelectric compound I states in heme proteins.

We further point out from our investigations that the use of single-crystal microspectrophotometry on these heme protein species is essential for correct determination of the state after the crystallographic data collection with synchrotron radiation. The Mb compound II and resting ferric Mb are both influenced rather quickly by the radiation but, unlike HRP, change to states that are quite unstable upon heating, resulting in the original state. For ferric Mb the reduced form is probably a low spin ferrous state with ligated hydroxide/water. To further clarify the states of the intermediates described above it should be interesting to perform single-crystal Mössbauer, EXAFS, and other spectroscopic analyses before and after x-ray radiation.

For Mb no large structural movements were observed except for the small changes in the heme region upon reaction with hydrogen peroxide. This seems to be a general feature of the peroxidase reaction cycle, the catalase reaction cycle, and the hydrogen peroxide. This seems to be a general feature of the compound II states and the isoelectric compound I states in heme proteins.

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