X-ray Absorption Studies of Myoglobin Peroxide Reveal Functional Differences between Globins and Heme Enzymes†

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ABSTRACT: X-ray absorption studies of myoglobin peroxide show that although it is not identical with compound I or II of horseradish peroxidase [Chance, B., Powers, L., Ching, Y., Poulos, T., Yamazaki, I., & Paul, K. G. (1984) *Arch. Biochem. Biophys. 235*, 596–611], it has some structural features in common with both. As seen in compound I, the Fe–O distance is short, but the iron–pyrrole nitrogen distance is contracted with a longer iron–histidine distance like compound II. The iron has a higher oxidation state than Fe^{3+}, suggesting an oxyferryl ion type species. Comparison of the structures of various peroxidase and myoglobin compounds points out systematic differences that may explain the catalytic activity of the π cation radical as well as some of the differences between globins and heme enzymes.

Heme protein chemistry has evolved from the study of the optical (Keilin & Hartree, 1937; Chance, 1943), magnetic (Theorell et al., 1952), and kinetic (Chance, 1952a,b) properties of heme compounds to the explanation of these properties through precise structural analysis. X-ray crystallographic analysis of myoglobin (Ten Eyck, 1979), catalase (Murthy et al., 1981), and cytochrome *c* peroxidase (ccp)[1] (Poulos et al., 1980) has provided clues as to how nature has utilized the porphyrin structural element in various ways to achieve diversity of function. In a recent paper, Chance et al. (1984) using X-ray absorption spectroscopy have proposed specific structures for the intermediates of horseradish peroxidase (hrp), compounds I and II. These results, in conjunction with the data in this paper, provide evidence for the following mechanism for the hrp and myoglobin reactions, expanded from that of Schonbaum and Chance (1976). The heme proteins provide two reducing equivalents to H_2O_2, forming water, and are rereduced by two one-electron steps to native enzyme, as in hrp, ccp, and lactoperoxidase, or by one two-electron step, as in catalase (Chance et al., 1984; Poulos & Finzel, 1984):

$$X\text{–}E\text{–}Fe^{3+} + HOOH \xrightarrow{k_1} X^{+}\text{—}E\text{—}Fe^{4+}{=}O + H_2O \xrightarrow[e^-]{k_3}$$

$$X\text{–}E\text{–}Fe^{4+}\text{–}O \xrightarrow[e^-]{k_5} X\text{–}E\text{–}Fe^{3+} + H_2O$$

where X^+ represents the hrp porphyrin radical or an oxidized myoglobin.

In the first reaction step, one reducing equivalent comes from the heme iron resulting in an Fe^{4+} state, while the other electron comes from a porphyrin radical as in hrp, chloroperoxidase, and lactoperoxidase or an amino acid radical as in ccp (Chance et al., 1984; Poulos & Finzel, 1984). The peroxide bond splits, and water is released with one oxygen atom remaining bonded to iron. This mechanism does not presume how the peroxide bond splits, whether the cleavage is homolytic or heterolytic. Myoglobin, like the peroxidases, reacts with H_2O_2 forming an oxidized enzyme intermediate, called myoglobin peroxide. Although this intermediate has been considered a model for the peroxidase compounds (George & Irvine, 1952, 1956), myoglobin does not complete the reaction cycle and is not a true peroxidase. The reaction of myoglobin is nonenzymatic in nature as the protein is altered through repeated cycles of reaction with H_2O_2 (Kelso et al., 1963).

Any differences in the structure of the myoglobin intermediate as compared to the structure of the hrp intermediates might provide clues about the fundamental differences between heme proteins as oxygen carriers (globins) and heme enzymes (peroxidases). For these reasons, we have studied the myoglobin peroxide compound by X-ray absorption spectroscopy. Our results confirm the idea that the iron in myoglobin peroxide has a higher oxidation state than ferric (met)myoglobin with an iron absorption edge shifted to higher energy. The shift is nearly as much as that measured for the ferrous to ferric myoglobin transition and is similar to the shift seen for compounds I and II when they are compared to native horseradish peroxidase. In addition, the EXAFS show iron closely bonded to one oxygen atom similar to compound I of hrp while the iron–pyrrole nitrogen average distance is much shorter.

Our results, in conjunction with the structural information provided by Chance et al. (1984) for horseradish peroxidase suggest that the catalytic activity of the hrp porphyrin radical depends on the proximity of the histidine ligand to the iron atom.

Experimental Procedures

Myoglobin was purified from Sigma horse type II with gel filtration and ion-exchange methods (Powers et al., 1984). The

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[1] Abbreviations: *c*, speed of light; ccp, cytochrome *c* peroxidase; eV, electronvolts; *E*, X-ray energy; E_0, iron edge energy or threshold; EXAFS, extended X-ray absorption fine structure; GeV, gigaelectronvolts; hrp, horseradish peroxidase; keV, kiloelectronvolts; LFIR, ligand field indicator region; N_p, pyrrole nitrogen; N_e, proximal histidine nitrogen.

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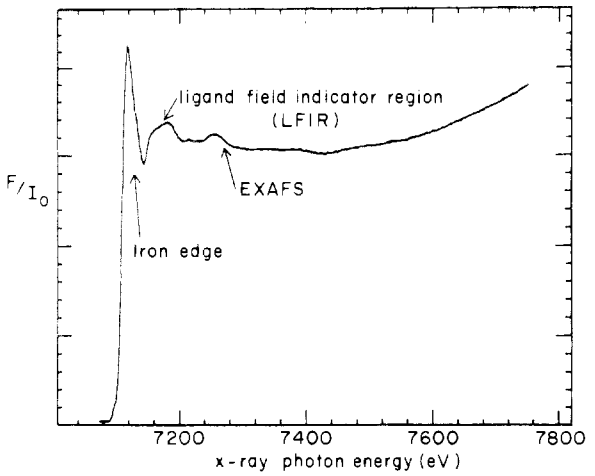

FIGURE 1: X-ray absorption spectrum of myoglobin peroxide with linear background subtraction.

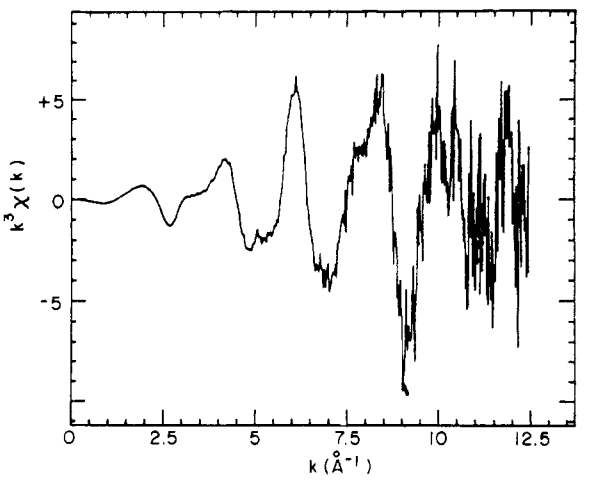

FIGURE 2: EXAFS of myoglobin peroxide. Fluoresence amplitude normalized to one absorbing atom and multiplied by k^3 vs. k.

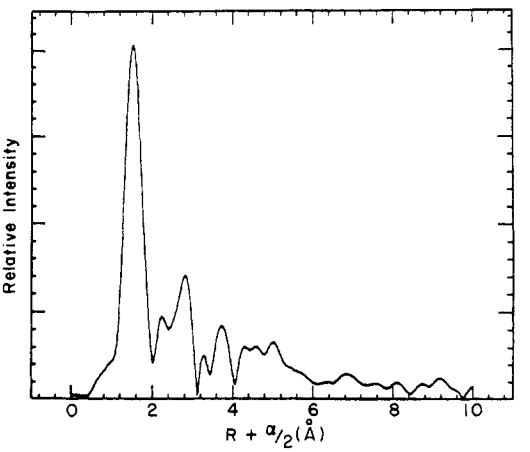

FIGURE 3: Fourier transform of myoglobin peroxide EXAFS data.

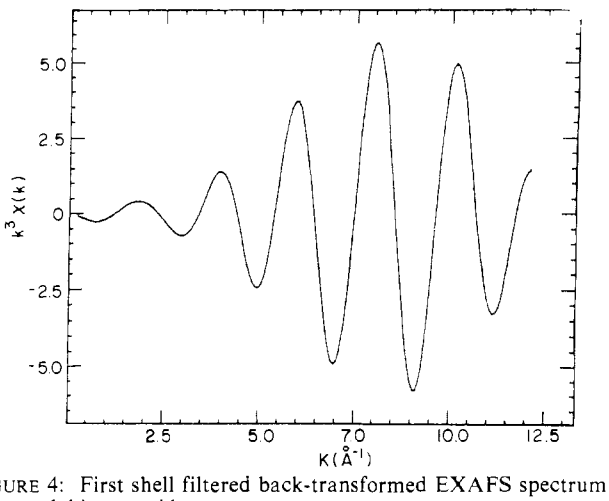

FIGURE 4: First shell filtered back-transformed EXAFS spectrum of myoglobin peroxide.

protein was concentrated by ultrafiltration to 3 mM and stored at −80 °C until use. Myoglobin peroxide was prepared by the addition of 2 mol of H_2O_2/mol of myoglobin at 4 °C in the X-ray sample holder, with a total volume of 175 μL in 30% ethylene glycol. The sample was stirred and cooled rapidly to stabilize the complex and stored in liquid N_2 until use. Reflectance spectrophotometry allowed the recording of optical changes in situ before and after X-ray exposure (Chance et al., 1983). The spectra showed the conversion from metmyoglobin to be complete (George & Irvine, 1956; Kelso et al., 1963), and no change was seen after the data collection was completed. A portion of the protein was oxidized in this reaction, but apparently, this does not alter the heme environment (Kelso et al., 1963).

X-ray absorption data were collected at the Stanford Synchrotron Radiation Laboratory under dedicated operation. Beamline II-2 was used with a previously described experimental setup (Powers et al., 1981). All experiments were carried out at 170 K. Data having 2–3-eV resolution were recorded by counting at a specified energy, E, for 2 s and incrementing the energy by 1 eV to the end of the scan for EXAFS studies. Edge studies were carried in the same fill with the V slits raised so the total flux was reduced by 75%. This gave a reproducibility of ±0.5 eV. Metmyoglobin was used as a standard to account for any changes in the monochromator. Count rates averaged 350 000 cps, and the total exposure time of the samples used was 5–7 h.

Eleven data scans were averaged, and the background was normalized by linear subtraction as shown in Figure 1. This spectrum was manipulated in various ways to make it suitable for comparison with model compound spectra (Chance et al., 1983; Powers et al., 1981; Peisach et al., 1981). First, several data scans were omitted before averaging if they contained serious beam glitches, freeze marks, or abrupt synchrotron noise. Second, the contribution of iron as an "isolated atom" was substracted from the spectra, and third, the fluoresence amplitude was multiplied by k^3, where $k = [(E - E_0)^{1/2}]/C$ and $C = h/[(2\pi)(2m_e)^{1/2}]$. This cancels the approximate $1/k^3$ dependence of the data and equalizes the contributions at high and low k.

The data are then plotted as a function of k as shown in Figure 2. The different ligand shells can be isolated by a Fourier transform of the data as shown in Figure 3. The first shell ligands have the highest electron density, and with a Fourier filter of 1.4 Å full width half-maximum, this contribution can be isolated and after back-transformation (Figure 4) can be compared with known iron models that have been treated in the same manner, as described below. Heme compounds are known to have up to six ligands bound to iron with four nitrogen atoms provided by the heme and one or two axial ligands, one usually a nitrogen of histidine from the peptide backbone. We used as comparable models bis(imidazole)-(5,10,15,20-tetraphenylporphinato)iron(III) chloride (Chance et al., 1983) and iron(3+) acetylacetonate (Iball & Morgan, 1976), the former having six N's at an average distance of 1.986 Å, which served as a model for Fe–N contributions, while the latter served as a model for Fe–O, with six O's at

Table I

	model	N	r	$\Delta\sigma^2$	ΔE_0	R^2
A	Fe–N	5	2.00	2.1×10^{-3}	1.7	1.6
	Fe–O	1	1.66	-5.2×10^{-3}	−0.9	
B	Fe–N	5	2.00	5.0×10^{-3}	0.1	6.4
	Fe–N	1	2.11	9.6×10^{-3}	8.5	
C	Fe–N	4	1.98	7.8×10^{-3}	−1.2	2.8
	Fe–N	2	2.09	9.6×10^{-3}	9.5	
D	Fe–N	4	2.05	6.5×10^{-3}	3.0	3.6
	Fe–N	2	1.95	9.1×10^{-3}	−8.0	
E	Fe–N	4	1.98	5.2×10^{-3}	1.5	1.4
	Fe–N	1	2.11	8.2×10^{-3}	2.0	
	Fe–O	1	1.66	8.0×10^{-3}	2.8	
F	Fe–N	4	2.05	1.6×10^{-3}	−8.6	20
	Fe–N	1	2.11	*−12.0*	*31.3*	
	Fe–O	1	1.66	2.8×10^{-3}	*−19.3*	
G	Fe–N	4	1.98	3.2×10^{-3}	5.6	1.8
	Fe–N	1	1.66	-5.6×10^{-3}	8.2	
	Fe–O	1	2.11	-3.0×10^{-3}	*−17.4*	
H	Fe–N	4	2.05	2.6×10^{-3}	−8.9	20
	Fe–N	1	1.66	4.3×10^{-3}	*−13.3*	
	Fe–O	1	2.11	*-1.4×10^{-2}*	−2.2	

an average distance of 1.99 Å.

The amplitudes and phases of these model compounds are then used to represent the contributions of two atom types in a nonlinear least-squares fitting program (Lee et al., 1981). Each atom type is represented by four parameters: N, r, $\Delta\sigma^2$, and ΔE_0, which are the number of scatterers, their average scattering distance, and the changes in Debye–Waller factor and edge energy, relative to the model compounds. The goodness of fit is described by the sum of residuals squared (R^2), and the correlation and Hessian matrices are evaluated. The correlation matrix gives the correlation of each parameter to every other parameter in the fit while the Hessian matrix provides eigenvectors for directional change; i.e., eigenvectors corresponding to small eigenvalues indicate directions along which little change in the minimum is observed for large changes in the corresponding parameters.

The data set shown in Figure 3, back-transformed with a filter of 1.4 Å full width half-maximum and a Δk from 3.5 to 12, contains seven to eight independent degrees of freedom (Lee et al., 1981). Since N and $\Delta\sigma^2$ are highly correlated and ΔE_0 is slightly correlated to r, these data can easily support the two atom type fit. Constraining the number of scatterers to their chemically known values and using amplitude ratios of 4/2 and 5/1 reduce the number of unknown parameters to six. A search of parameter space is afforded by setting different initial conditions of distance and searching for minima in R^2, which are physically and chemically reasonable. The ΔE_0 has been shown both experimentally (Powers et al., 1979) and theoretically (Lee et al., 1981) to have an absolute value less than or equal to about 10 eV for compounds with the same absorbing and scattering atoms. Solutions that exhibit large ΔE_0 (above 10 eV) are chemically unreasonable and indicate the distance to which it is correlated is in error.

We define $\Delta\sigma^2$ as σ^2 of the model minus σ^2 of the unknown so that as $\Delta\sigma^2$ becomes more negative, the bond in question is seen to be *looser*. But, since N and σ^2 are highly correlated and N is held fixed, all possible errors in N are contained in $\Delta\sigma^2$ and the cross correlation of the parameters is high. This means that individual $\Delta\sigma^2$ values reported in Table I are not necessarily reflective of the bond distance in question but of the fit as a whole. As such, the reasonable limits for $\Delta\sigma^2$ are from -1×10^{-2} to 1×10^{-2}. Values that are several times this absolute value are also unreasonable and chemically and mathematically represent the fitting program attempting to decrease or amplify the amplitude of that contribution when N has been fixed. The results of such a search for myoglobin peroxide are shown in Table I.

These solutions contain N/r^2 averages of the three potentially different contributions found in hemes, i.e., four pyrrole nitrogens and two axial ligands. The 5/1 amplitude ratio fits are intended to reveal possible axial distances while the 4/2 ratios are intended to reveal possible Fe–N_p scatering distances. Comparison of the fixed-amplitude solutions reveals the approximate distances for each of the three kinds of scatterers present.

In order to determine if these approximate distances, in combination, are indeed contained in the data and not artifacts of the fitting procedure or to distinguish between combinations of these approximate distances, a three atom type consistency test was used. This procedure is not fitting but a test of the compatibility of the possible different contributions. In the consistency test, both r and N are held fixed (the choice of r determined by the two-atom procedure); thus, only those parameters that are correlated to those held fixed ($\Delta\sigma^2$ and ΔE_0) are allowed to vary. This test also has six unknowns, like the two atom type procedure, but since both N and r are fixed, *all* the unknowns are correlated to the fixed parameters and to each other.

Unreasonable values for $\Delta\sigma^2$ and ΔE_0 or an R^2 greater than the best of those observed for the solutions in the two atom type fitting procedure indicate incompatible distance combinations. In order to assure a reasonable combination constituted a true minimum, each distance was allowed to vary one at a time (a test with less than seven unknowns), and the best combination distances remained ±0.03 Å from the initial estimates. These results for myoglobin peroxide are shown in Table I. A complete description of this procedure with examples and comparisons to results obtained by crystallographic studies is given in Powers et al. (1984) and Woolery et al. (1985). In each case, the results of this procedure are found to be identical within the errors with those reported by the crystallographic studies, including the results from small-molecle studies where the reported error is comparable to that of this procedure.

The second shell was analyzed by filtering the higher shell data in the Fourier transform with a similar window. These back-transformed data were fit to models with the two atom type procedure, where the partially resolved second and third shells of the heme and proximal histidine contributions represent a single-atom type and iron(3+) acetylacetonate was

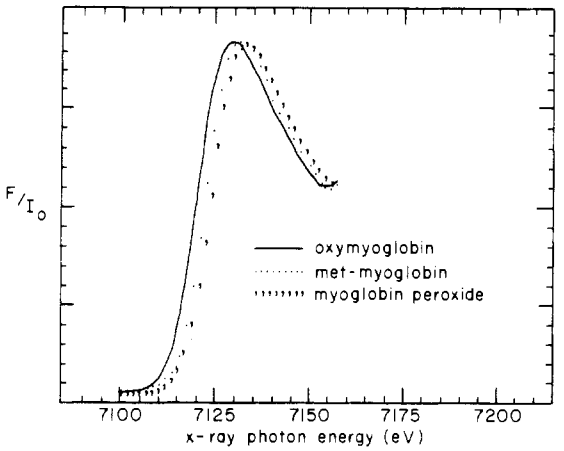

FIGURE 5: X-ray edge spectra of oxymyoglobin (—) (Fe^{2+}), metmyoglobin (···) (Fe^{3+}), and myoglobin peroxide (,,,).

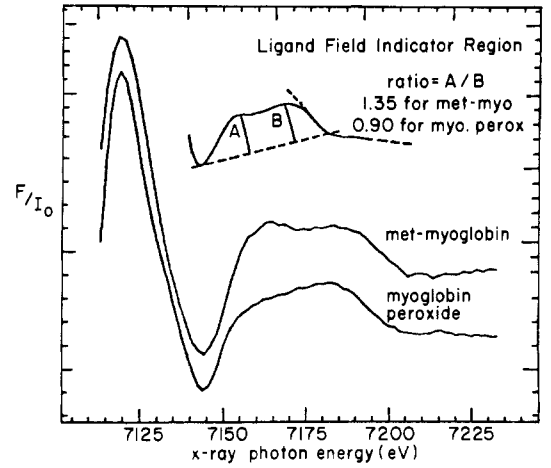

FIGURE 6: Ligand field indicator region (LFIR) of metmyoglobin compared to that of myoglobin peroxide.

used as a second atomic type to model the addition of an oxygen atom.

Results

The edge spectrum of myoglobin peroxide was compared with the edge spectra of metmyoglobin (Fe^{3+}) and oxymyoglobin (Fe^{2+}) (L. Garcia-Iniquez, L. Powers, and B. Chance, unpublised observations). The edge shift corresponding to a valence change of one electron is 1.5 ± 0.5 eV as estimated by the difference between the oxymyoglobin and the metmyoglobin edges (Figure 5). The edge of myoglobin peroxide is 1.0 ± 0.5 eV higher in energy than metmyoglobin and 2.5 ± 0.5 eV higher than oxymyoglobin. This indicates that the myoglobin peroxide complex has an iron atom oxidized by up to one electron relative to the Fe^{3+} state.

The ratio of peaks in the ligand field indicator region (LFIR) for myoglobin peroxide and metmyoglobin is shown in Figure 6. The ratio is reduced by one-third in myoglobin peroxide compared with metmyoglobin. This spectral region has been proposed to correlate with spin state and Fe–N_p distances in ferric and ferryl hemoprotein compounds (Chance et al., 1984). In fact, Fourier-transform analysis of first shell vs. higher shell contributions shows that changes in this region are correlated with changes in backscattering from higher shell ligands (M. Chance, unpublished observations). Therefore, this region cannot be used to indicate directly first shell distances but provides an internal spectral assignment for each scan. Increases in metmyoglobin byproduct in the sample would be directly reflected in an increased ratio for that scan. The expected value of an $S = 1$ species is 0.84; the reported value of 0.90 is within the error (±0.03) and indicates no metmyoglobin contamination. In summary, while the LFIR measurements' correlation with specific interatomic distances is still being investigated, its use in verifying the sample identity and authenticity in conjunction with optical measurements is important.

The two atom type fitting procedure found four reasonable solutions, shown in Table I. The 5/1 amplitude ratio fits gave axial distances of 1.66 and 2.11. The 4/2 fit gave two solutions, the larger of which was not consistent with the much lower average distance seen in the 5/1 fits. Various combinations of these distance solutions were tested in the three-atom procedure. As shown in Table I, solution E is the only reasonable combination as any other grouping violates chemical reasonableness as indicated in italic type. In addition, when solution E was iterated, only it remained close to its two scatterer solutions, reporting 1.98 ± 0.02 Å for Fe–N_p, 2.11 ± 0.02 Å for Fe–N_e, and 1.69 ± 0.03 Å for Fe–O. The error is calculated by changing each distance individually until the R^2 increased 2-fold. It should be noted that solutions A and E are the same solution. The $1/r^2$ average of four contributions of 1.98 and one contribution of 2.11 equals five contributions of 2.00.

Solution F, with the large Fe–N_p, is clearly not in the data. Solution G, which switches the axial ligand models, shows the sensitivity of the test in identifying the atomic type. The ΔE_0 shows the combination is clearly inferior to that of solution E. The amplitude differences between O and N are very small but enhanced at low temperature. The two-atom test cannot distinguish the atomic types as the two solutions comparable to A and B (with Fe–N and Fe–N models for the distances in A and Fe–N and Fe–O models for the distances in B) are nearly identical. Combinations not shown include the possible solutions with the axial distances equal, i.e., Fe–O and Fe–N at 1.66 or 2.11 Å. They are not shown because the R^2 values are from 50 to 100 and the $\Delta\sigma^2$ and ΔE_0 values are huge.

Comparison of the Fourier transforms shown in Figure 7 shows that the higher shell of myoglobin peroxide resembles metmyoglobin more than oxymyoglobin. This suggests that myoglobin peroxide has no oxygen atom in these partially resolved shells (unlike an iron peroxide) and is more like metmyoglobin having only one oxygen bound, which is found in the first shell. These results were confirmed by representing the higher shells of metmyoglobin and oxymyoglobin as a single contribution and fitting them to the higher shells of myoglobin peroxide in a single-atom procedure. The R^2 was 2.3 for the fit with met and 9.1 for oxy. By use of the two atom type procedure with the Fe–O model as the second contribution, the addition of an oxygen atom to the fit produced an unreasonable Debye–Waller factor. When that contribution's coordination amplitude was allowed to vary, it went to zero. In general, to provide solid evidence for the existence of an oxygen in the second shell, this kind of test requires that R^2 decreases 10-fold in response to the addition of the oxygen atom. These results imply the absence of any second shell oxygen, like those found in hrp compound III and oxymyoglobin.

Discussion

A variety of techniques, including optical (Keilin & Hartree, 1937; Chance, 1943), EPR (Schulz et al., 1979; Rutter et al., 1983), NMR (LaMar et al., 1982), resonance Raman (Rakshit & Spiro, 1974, 1976; Teraoka & Kitagawa, 1980), ENDOR (Roberts, et al., 1981), Mössbauer (Schulz et al., 1979; Rutter et al., 1983; Maeda et al., 1971), and X-ray

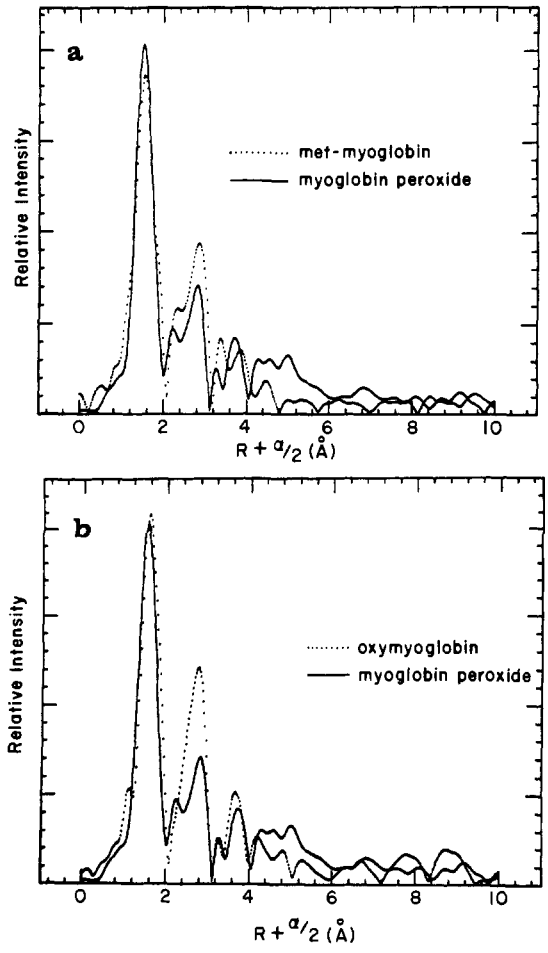

FIGURE 7: (a) Fourier transforms of metmyoglobin (···) EXAFS compared to that of myoglobin peroxide (—). (b) Fourier transform of oxymyoglobin (···) EXAFS compared to that of myoglobin peroxide (—).

absorption spectroscopy (Chance et al., 1984), have been used to establish the peroxidase reaction sequence. In addition, X-ray crystallographic data are available for myoglobin and ccp, while some sequence data are available for the other peroxidases. For example, Mössbauer and X-ray absorption edge data have confirmed the idea that the heme iron atom donates one electron in the formation of hrp compound I (Chance et al., 1984; Schulz et al., 1979), as well as in myoglobin peroxide (Maeda et al., 1971). Resonance Raman evidence and X-ray edge studies have also indicated that hrp compound II has an iron atom in the Fe^{4+} state (Chance et al., 1984; Rakshit & Spiro, 1976). In addition, the porphyrin radicals proposed for the hrp, chloroperoxidase, and lactoperoxidase compound I's are supported by their characteristic green optical absorption spectra (Dolphin et al., 1971). Ccp compound ES having an amino acid radical and myoglobin peroxide having no stable porphyrin radical species are red. Compound II of hrp, which is Fe^{4+}, also is red, having its porphyrin radical filled before the iron is reduced to the native Fe^{3+} level in the second reduction step. Our X-ray absorption results support the idea that one oxygen atom is bonded to iron in myoglobin peroxide, similar to the structure proposed for hrp compound I by Chance et al. (1984) seen in Figure 8. Recent resonance Raman evidence (Sitter et al., 1985) supports this short bond distance for Fe–O in myoglobin peroxide. The short bond distance implies a bond similar to Fe=O, as seen in model compounds (Penner-Hahn et al., 1983).

Therefore, the major similar feature of heme–peroxide reactions is the formation of an oxyferryl ion, or oxo species, as first proposed by George (George & Irvine, 1952, 1956). In addition, the major distinguishing features are the source of the second electron equivalent and the rate of reaction with H_2O_2. The different peroxidases provide the second electron equivalent in different ways, and myoglobin, which is not a catalyst, cannot form the stable, reversible radical state that is necessary for the steps after compound I formation. In

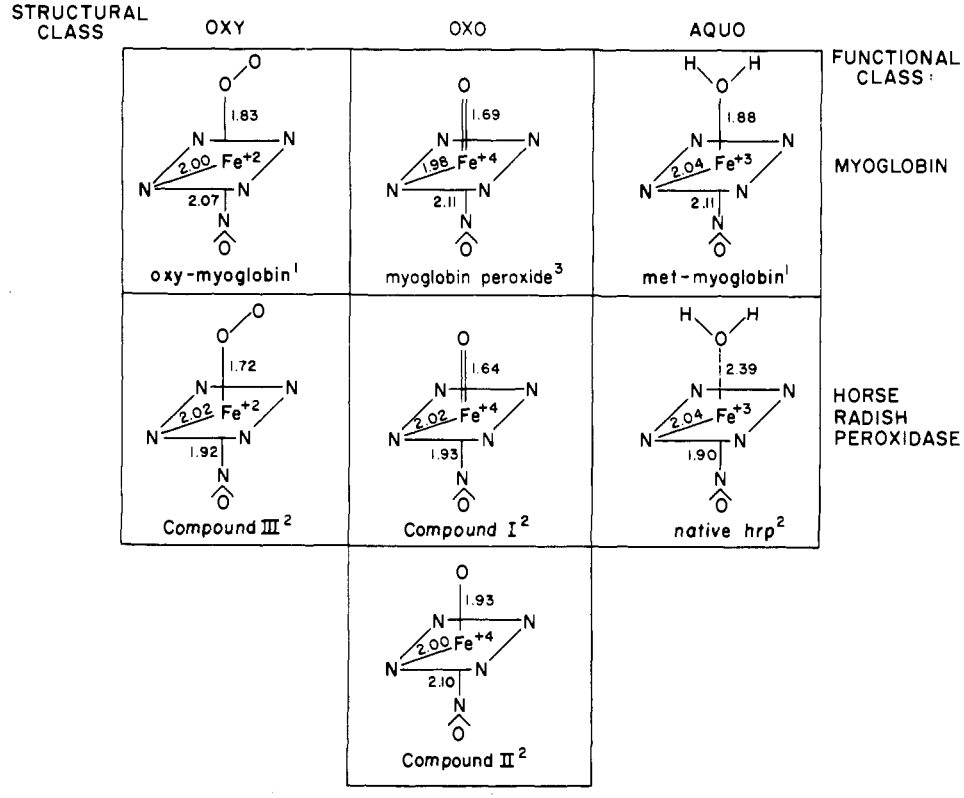

FIGURE 8: Structures of myoglobin and peroxidase intermediates as shown by EXAFS. All Fe–N_p distances are ± 0.01 Å except myoglobin peroxide (±0.02 Å). All other distances are ±0.02 Å. (1) From Powers et al. (1984). (2) From Chance et al. (1984).

myoglobin, Winfield has suggested that the radicals formed disproportionate to form an irreversibly altered protein, perhaps with carbon–carbon double bonds in the heme packet (Kelso et al., 1963). Myoglobin is also very slow to react with peroxide compared to the peroxidases. But, this difference may be due to differences in the particular amino acids found in the heme pocket (see below).

The structure of myoglobin peroxide (Table I and Figure 8) shows that it is different from both compound I and compound II of hrp. Specifically, compound II and myoglobin peroxide are similar in proximal histidine and Fe–N_p distance but different in Fe–O distance. Compound I, on the other hand, is quite similar to myoglobin peroxide in Fe–O distance. The proximal histidine is close to iron in compound I at 1.93 ± 0.02 Å and more distant in myoglobin peroxide and compound II at 2.10 ± 0.02 Å. Compound I is intermediate spin (Theorell et al., 1952) with an expanded Fe–N_p distance of 2.02 ± 0.01 Å while compound II and myoglobin peroxide are contracted at 2.00 ± 0.01 and 1.98 ± 0.02 Å, respectively. The resonance Raman measurements on myoglobin peroxide and compound II of hrp by Sitter et al. (1985) and Terner et al. (1985) support the structures for the pyrrole nitrogens we propose. Specifically, the polarized line at 1580–1600 cm^{-1} implies a C_t–N distance of 1.98 Å for myoglobin peroxide and 1.99 Å for hrp compound II. Unfortunately, C_t–N and Fe–N_p are only directly correlated if the position of iron with respect to the heme plane is constant. Since this is not known for these compounds, the agreement between the two techniques may be fortuitous. However, if the iron is in the plane in both cases, the agreement is exact within the respective error. In addition, some investigators disagree with the Fe–O distance reported for hrp compound II by Chance et al. (1984). They suggest alternatively that the Fe–O bond is short, like in hrp compound I (Penner-Hahn et al., 1983; Terner et al., 1985).

Rutter et al. (1983) have assigned the hrp compound I radical to an A_{2u} molecular orbital. We find such an assignment plausible compared to an A_{1u} assignment, since the latter has no overlap with the iron orbitals. The iron–ligand distances in compounds I and II of hrp are quite different, consistent with a change in A_{2u} orbital occupancy. If the compound I radical was from an A_{1u} orbital, little or no change in iron–ligand distances would be expected when that orbital became fully occupied after reduction. Myoglobin peroxide, having no stable radical, resembles compound II in this sense, having a fully occupied A_{2u} molecular orbital and a short Fe–N_p distance.

As can be seen in Figure 8, similarities exist between other myoglobin compounds and peroxidase compounds. For example, oxymyoglobin and compound III of hrp have similar oxidation states, similar Fe–N_p distances, LFIR ratios equal within the error, and dioxygen ligands, but with the oxygen being 0.11 ± 0.04 Å closer in compound III. Metmyoglobin and native horseradish peroxidase are also quite similar in heme structure. They are both high spin, with Fe–N_p distances of 2.04 Å and LFIR ratios of approximately 1.30. They have ferric iron and form oxo complexes when reacted with H_2O_2. The similarities of these heme classes, called oxy, oxo, and aquo, have been mentioned, but the differences are striking. Each of the compounds, with the exception of compound II, has Fe–N_e distances characteristic of peroxidases or myoglobins. The average distances are 1.92 ± 0.05 Å for the peroxidase compounds and 2.09 ± 0.04 Å for the myoglobin compounds, the average distances differing by 0.17 ± 0.05 Å. This histidine distance difference is also seen in oxyhemoglobin in a report by Shaanan (1982) using X-ray crystallography. He reports Fe–N_e distances of 1.95 ± 0.05 Å for the α chains and 2.06 ± 0.05 Å for the β chains of oxyhemoglobin. In short, the α chains resemble those of peroxidases while the β chains resemble those of myoglobin.

In further comparison, one sees that for metmyoglobin and native hrp (note native hrp is five-coordinate), an increase in Fe–N_e is accompanied by a decrease in Fe–O. In this case, Fe–N_p is unchanged within the error. For myoglobin peroxide and hrp compound I, however, the increase in Fe–N_e is accompanied by no measurable change in Fe–O. More striking is the *increase* in Fe–O for oxymyoglobin as Fe–N_e increases compared to hrp compound III. In these two cases, Fe–N_p is not equal within the error, the average distances changing at least 0.01 Å per pyrrole nitrogen. Crystallographic (Collman et al., 1974, 1975; Jameson et al., 1978a,b, 1980) and EXAFS (Woolery et al., 1985) results of model compounds have shown that exceptions to the trans effect can occur when the proximal imidazole is hindered. In summary, the electron distribution observed in these intermediates is a function of the interplay of the histidine and the iron and its relationship to the porphyrin plane affecting the orbitals of the sixth ligand.

The presence of a strategically situated charged group, present in the heme pocket above the sixth heme ligand position in the crystal structure of ccp, has been proposed as an important structural feature distinguishing peroxidase and globin-like proteins (Poulos et al., 1980, 1984). It is noteworthy that an arginine is conserved in this sequence position in all the peroxidases studied and that no comparable charged group exists in the myoglobin heme pocket (Poulos & Finzel, 1984). We agree with Poulos that it is plausible that the presence of this positive charge polarizes the O–O bond and is largely responsible for the faster rate of formation of the oxo (compound I like) species in peroxidases as compared to myoglobin, which has been reported since the work of Keilin and Hartree (1937). However, the closeness of the proximal histidine in hrp, relative to myoglobin, plays a significant role in the structures of the resultant oxo intermediates. When H_2O_2 reacts with hrp, the short Fe–N_e distance forces electron density to be delocalized in the heme, making Fe–N_p relatively large in compound I. In myogobin peroxide, the histidine distance is larger than that in compound I, and Fe–N_p is correspondingly shortened. NMR results have indicated that the iron–proximal histidine bond is different in hrp than in myoglobin (LaMar et al., 1982). Additionally, resonance Raman spectra show an anomalously high value for the reduced hrp Fe–N_e stretching mode compared to ferrous myoglobin, indicating a different bond for hrp (Teraoka & Kitagawa, 1980). Both these results have until now been interpreted as an increase in protonation of the histidine for the peroxidase. Poulos and Finzel (1984) have also pointed out an intricate hydrogen-bonding network for the proximal histidine in ccp. Compared to the myoglobin hydrogen bonds, they predict a more tightly hydrogen-bonded histidine for the peroxidase. A combination of these differences and the closeness of the histidine in hrp may play a role together in the stabilization of the radical state. It is possible that no stable porphyrin radical forms in myoglobin because the heme system is unable to delocalize electron density from the proximal histidine to stabilize an A_{2u} radical that might have formed. This conclusion is consistent with the structure of compound II. When the A_{2u} radical is filled by the one-electron reduction of compound I, the Fe–N_p contracts and the histidine distance expands similar to the distance found in myoglobin peroxide. However, the reaction kinetics of myoglobin with H_2O_2 (k_1

$\ll k_3$) are particularly inappropriate to the detection of a porphyrin radical, if it were formed.

It is interesting to note that native ccp has a short $Fe-N_p$ distance like hrp and catalase has a short Fe–O (Chance et al., 1984). Examination of the oxo intermediates of these enzymes should prove useful in our understanding of this structure–function relationship.

In summary, we provide direct evidence that in myoglobin peroxide the oxidation state of the iron is greater than Fe^{3+} and that one oxygen atom is present and bonded very close to the iron in the sixth position. This species is low-spin unlike its precursor, metmyoglobin, and has a contracted $Fe-N_p$ with an average distance of 1.98 Å. The evidence is consistent with an oxyferryl ion type species as first proposed by George (George & Irvine, 1952, 1956). We have also suggested that structural classes, termed oxy, oxo, and aquo, exist in the functional classes of horse radish peroxidase and myoglobin. These similar classes of compounds beg the question of protein vs. enzyme. The myoglobin proximal histidine distances for the three compounds are 0.17 ± 0.05 Å longer on average. This distance difference and the effect it has on the heme π-electron density may be largely responsible for the ability of hrp to form a stable porphyrin radical.

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Registry No. hrp, 9003-99-0; Fe, 7439-89-6; O_2, 17778-80-2; N_2, 17778-88-0.

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