An Extended X-ray Absorption Fine Structure Investigation of the Structure of the Active Site of Lactoperoxidase†

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Received September 15, 1992; Revised Manuscript Received December 31, 1992

ABSTRACT: Native lactoperoxidase, compound III, and the reduced forms (at pH 6 and 9) were studied using X-ray absorption spectroscopy (XAS). Native lactoperoxidase has four pyrrole nitrogen ligands at an average distance of 2.04 ± 0.01 Å, a proximal ligand at 1.91 ± 0.02 Å, and a sixth (distal) ligand at 2.16 ± 0.03 Å. Lactoperoxidase native enzyme has a first coordination shell structure that is similar to that of native lignin peroxidase [Sinclair, R., Yamazaki, I., Bumpus, J., Brock, B., Chang, C.-S., Albo, A., & Powers, L. (1992) *Biochemistry 31*, 4892–4900] and different from that of horseradish peroxidase [Chance, B., Powers, L., Ching, Y., Poulos, T., Schonbaum, G., Yamazaki, I., & Paul, K. (1984) *Arch. Biochem. Biophys. 235*, 596–611]. Similarly, lactoperoxidase compound III resembles lignin peroxidase compound III. The five-coordinated ferrous form was stable at pH 9, but at pH 6 it was rapidly converted to the six-coordinated form with a distal ligand at 2.18 ± 0.03 Å. No evidence typical of changes in spin state was obtained at the different pH values.

Lactoperoxidase (LPO),[1] a secreted glycoprotein with molecular mass ~77 000 Da (Carlstrom, 1969), of which 10% is carbohydrate (Sievers, 1981), is found in mammalian milk. LPO, along with related peroxidases in tears and saliva, forms part of an antimicrobial defense system (Reiter & Perraudin, 1991). Following reaction with a molecule of hydrogen peroxide, LPO is able to react with various substrates and catalyzes the peroxidation of endogenous thiocyanate to the antimicrobial hypothiocyanite ion in mammalian secretion systems (Hamon & Klebanoff, 1973). LPO forms compounds I and II during its catalytic cycle and ferrous derivatives such as reduced enzyme (ferrous form) and compound III (ferrous-oxy form) can also be produced (Kohler et al., 1988). Compound III is not involved in the catalytic cycle but its accumulation reduces the activity of the enzyme.

The mammalian peroxidases LPO, thyroid peroxidase, and intestinal peroxidase (IPO) exhibit optical spectra that are similar (Ohtaki et al., 1985). However, these spectra are different from those of typical plant or fungal peroxidases such as horseradish peroxidase (HRP) (Yokota & Yamazaki, 1977) or lignin peroxidase (LiP) (Renganathan & Gold, 1986). Even though the spectra of the animal peroxidases are similar, there are striking differences in the reactivity. LPO, like HRP, catalyzes the univalent oxidation of certain tyrosine analogues while thyroid peroxidase performs similar oxidations of these tyrosine analogues via a bivalent mechanism (Nakamura et al., 1985). Furthermore, differences in the reactivity of LPO and IPO have also been noted (Kimura & Yamazaki, 1979). Chloroperoxidase and myeloperoxidase are able to oxidize Cl^-, Br^-, and I^-, while LPO, HRP, and LiP are unable to oxidize Cl^- (Griffin, 1991; Neidleman & Geigert, 1986; Renganathan et al., 1987).

Although no crystal structure is available for LPO, the protein sequence of the main active fraction from cow's milk was recently determined (Cals et al., 1991). Many characteristics of the LPO heme and its derivatives are also known. Early composition analysis using pronase digestion (Sievers, 1979) suggested that LPO has a protoheme IX which is covalently bound to the protein through a disulfide bond; this was confirmed by Nichol et al. (1987). A number of spectroscopic studies revealed that LPO has a comparatively small heme pocket and a histidine imidazole at the fifth (proximal) coordination position of the heme iron (Sievers, 1980; Shiro & Morishima, 1986; Lukat et al., 1987). Resonance Raman spectroscopy confirmed that the heme active sites of LPO and its derivatives have a geometry that is similar to other peroxidases. Reczek et al. (1989) demonstrated that LPO compound II has a similar structure to cytochrome *c* peroxidase (CcP) ES complex but with stronger hydrogen bonding to a distal arginine. Optical absorption studies (Carlstrom, 1969; Sievers, 1980) of ferrous LPO revealed different spectra at neutral and alkaline pH. This behavior is similar to that of intestinal peroxidase, in which a five-coordinate form was stable at alkaline pH but changed to a six-coordinate form below pH 6.5 (Kimura et al., 1981).

In the work presented here, XAS was used to probe the active-site structure of native LPO and the ferrous and ferrous-oxy forms. XAS studies have revealed similarities and differences in the heme active-site structures of several peroxidases including HRP (Chance et al., 1984; Penner-Hahn et al., 1983, 1986; Chang et al., 1991), CcP (Chance et al., 1984, 1986a), catalase (Chance et al., 1984), and LiP (Sinclair et al., 1992). The results for LPO are compared to those for other peroxidases.

† Supported in part by NIEHS Superfund Grant ES 04922, the Savannah River Laboratory, Contract W89329-BA, and NIH Grant RR-01633.

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HRP, horseradish peroxidase; IPO, intestinal peroxidase; LFIR, ligand field indicator region; LiP, lignin peroxidase; LPO, lactoperoxidase; XAS, X-ray absorption spectroscopy.

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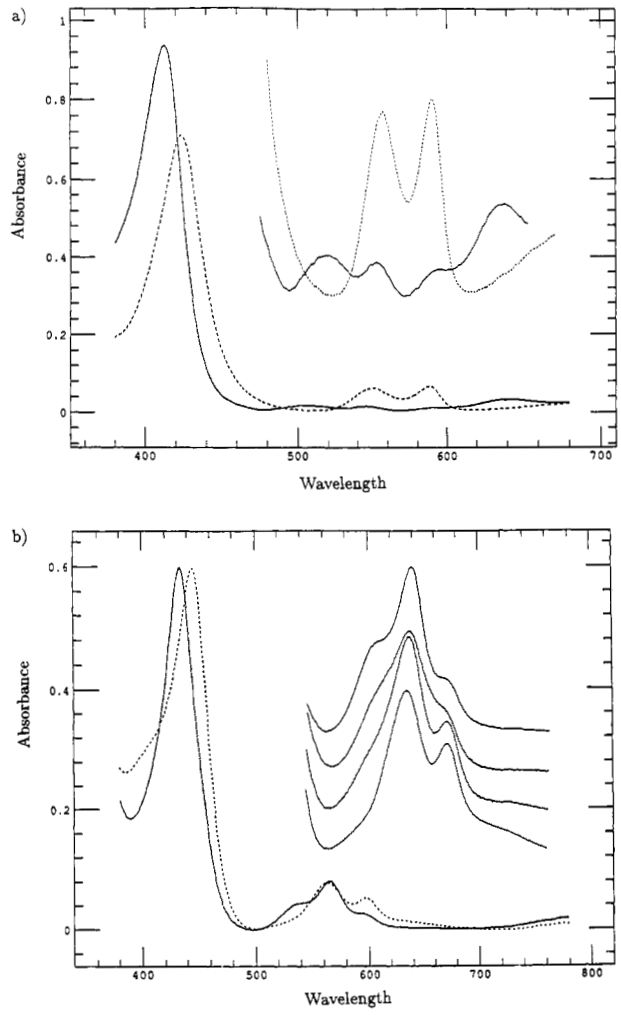

FIGURE 1: Optical spectra of lactoperoxidase. (a) Native LPO (—) and compound III (- - -). Inset shows the visible region with an absorbance scale expanded by a factor of 5. (b) Ferrous LPO at pH 9.0 (ferrous-445, - - -) and pH 6 (ferrous-434, —). Inset shows the changes in the visible region over time at pH 6.0: ferrous-445 form at t = 0, 3, and 10 min and ferrous-434 form at 20 min.

MATERIALS AND METHODS

Sample Preparation. LPO was purified from cow's milk (Rombauts et al., 1967) to an RZ value (A_{412}/A_{280} ratio) of 0.91. The concentration of LPO was determined spectrophotometrically $\epsilon_{mM^{-1}cm^{-1}}$ at 413 nm = 114 (Morrison & Hultquist, 1963). At low concentration, compound III was formed by reacting 10 μM native enzyme with 50 molar equiv of analytical-grade H_2O_2 in 10 mM phosphate buffer containing 40% ethylene glycol. The ferrous forms were prepared by adding solid sodium dithionite ($Na_2S_2O_4$) to 10 μM native enzyme in 10 mM carbonate buffer (pH 9.2) or phosphate buffer (pH 6.0). The purity of all samples was verified by UV–vis spectroscopy using the Soret, β, and α bands (Figure 1).

Highly concentrated samples of LPO (native enzyme and compound III) for XAS experiments were prepared at 1.75 mM in 40 mM phosphate buffer at pH 7 containing 40% ethylene glycol. Manthey et al. (1986) showed that no low-spin components are observed using EPR in the presence of 30% ethylene glycol. Because of the difficulty in obtaining a homogeneous preparation at high concentrations using lower ratios of H_2O_2 to LPO, compound III was prepared at a 200:1 molar ratio of H_2O_2 to LPO. A catalase-like activity results in frothing of the sample at millimolar concentrations due to oxygen evolution. The material was centrifuged briefly to eliminate bubbles prior to loading of the XAS sample holder. The optical spectra of native enzyme and compound III are shown in Figure 1a. Ferrous forms were prepared by adding excess solid sodium dithionite to 1.75 mM native enzyme in either 40 mM sodium phosphate buffer at pH 6 or 40 mM sodium carbonate buffer at pH 9.2. These enzyme preparations had previously been surface-purged with N_2 gas for at least 15 min. The ferrous form with the Soret band at 434 nm (ferrous-434) was prepared at pH 6.0 and was identified using UV–vis spectroscopy of a diluted aliquot 20 min after the dithionite was added (Figure 1b). This material was then frozen in liquid N_2. The ferrous form with the Soret band at 445 nm (ferrous-445) was prepared at pH 9.2, identified using UV–vis spectroscopy of a diluted aliquot after 1 min, and then frozen in liquid N_2 (Figure 1b). The samples were also examined using reflectance spectroscopy prior to X-ray exposure.

All samples were maintained at ~–100 °C during XAS data collection. After X-ray exposure, the integrity of native enzyme, compound III, and ferrous forms was checked by reflectance spectroscopy. Small portions of each sample were also thawed and diluted for transmission spectroscopy. The spectra indicated that the compounds were stable during data collection. Compound III, when thawed at room temperature, converted to native form. After XAS data collection, long-term storage of the ferrous forms in liquid nitrogen did produce some changes in the spectra.

Data Collection and Analysis. XAS data were collected at the Stanford Synchrotron Radiation Laboratory on beamline 2-1 and at the National Synchrotron Light Source on beamline X9, both equipped with Si(111) monochromator crystals. Data with a resolution of 2–3 eV were recorded in fluorescence mode using a phosphorescent phototube detector and manganese filters (Khalid et al., 1986). These data were then analyzed by methods described previously (Powers et al., 1981, 1984; Lee et al., 1981). Single scans were averaged to enhance the signal-to-noise ratio. The ligand field indicator region (LFIR) parameter was calculated according to Chance et al. (1984). Signal averaging was followed by background subtraction and k^3 multiplication, where k is the photoelectron wave vector (Figure 2). The EXAFS modulations shown in Figure 2 were then Fourier-transformed (Figure 3). Generally, modulations from k = 0 to ~12.5 Å were used for the analysis. Similar data were collected for three model compounds [Fe^{3+}-bis(imidazoletetraphenylphinato)chloride (Collins et al., 1972), Fe^{2+}-(acetylacetonate), and Fe^{3+}-(acetylacetonate) (Fe^{+2}acac and Fe^{3+}acac, respectively) (Iball & Morgan, 1969)]. The scattering signal derived from the first Fourier transform peak of each enzyme was fitted to that of the model compounds using two sets of scattering atoms. Each atom type in the fitting procedure is represented by an average distance (r), an amplitude factor containing the number of ligands (N), a change in Debye–Waller factor ($\Delta\sigma^2 = \sigma^2_{model} - \sigma^2_{unknown}$) and a change in threshold energy ($\Delta E_0 = E_{model} - E_{unknown}$). Since the amplitude factor is highly correlated with $\Delta\sigma^2$, the N values were held constant at their known values (Peisach et al., 1981). By use of a two-atom-type nonlinear least-squares fitting procedure, the amplitude ratios were fixed at 4/2 to isolate the Fe–N_p (average iron to pyrrole nitrogen distance) or at 5/1 to isolate the Fe–O/N (iron to distal ligand distance) or Fe–N_ϵ (iron to proximal histidine nitrogen distance). Several possible solutions were judged to be different only if the $\sum R^2 > \sum R^2_{min}(1 + 1/\phi_f)$, where $\sum R^2_{min}$

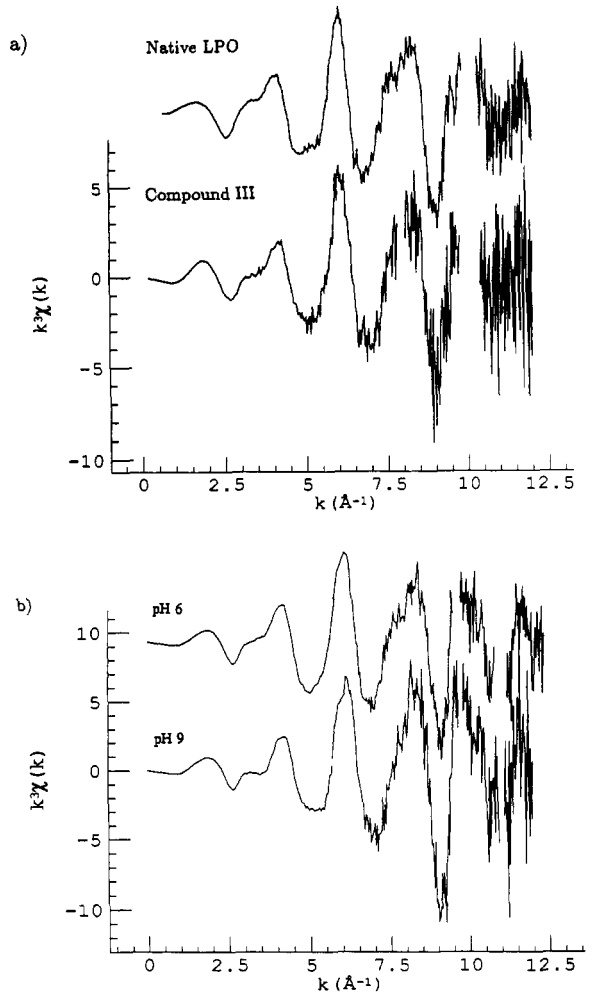

FIGURE 2: EXAFS data normalized to one Fe atom, background-substracted, and multiplied by k^3. (a) Native LPO (top) and compound III (bottom). (b) Ferrous-434 form (top) and ferrous-445 form (bottom).

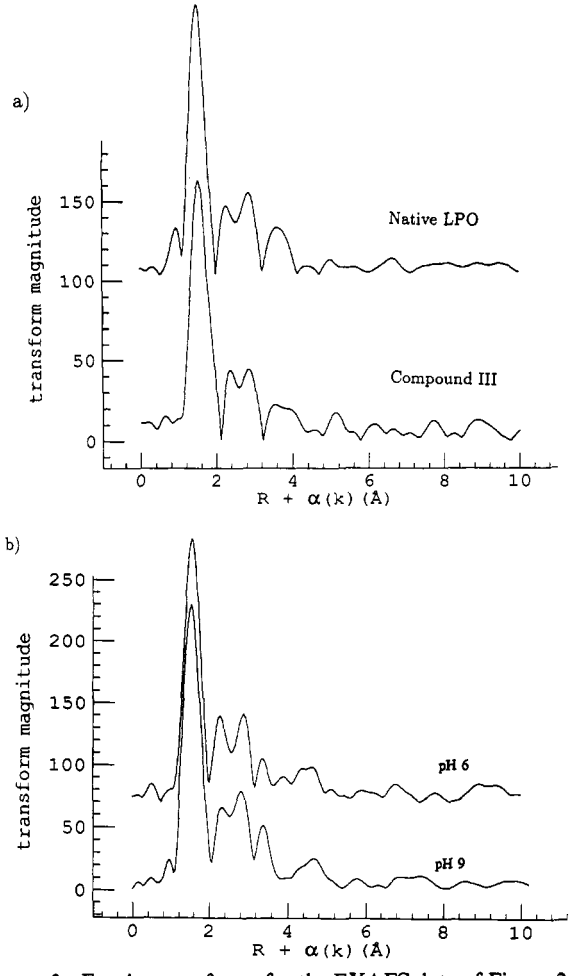

FIGURE 3: Fourier transforms for the EXAFS data of Figure 2. (a) Native LPO (top) and compound III (bottom). (b) Ferrous-434 form (top) and ferrous-445 form (bottom).

is the minimal sum of residuals for a physically reasonable solution and $\phi_f = \phi_d - p$, where p is the number of variables in the fit and ϕ_d is maximum number of degrees of freedom in the filtered data. The variable ϕ_d was estimated by $2\Delta\omega\Delta k/\pi$, where $\Delta\omega$ was the full width at half-maximum of the filter window and Δk is the length of the data used. Thus the criterion for the data presented here is that $\sum R^2 > (1.4)\sum R^2_{min}$. After solutions were found for each contribution to the first shell [Fe–N_p, Fe–O(N), and Fe–N_ϵ], a three-atom-type consistency test was carried out using all three sets of scatterers, with r and N held constant for each set of scatterers, in order to verify that the three distances together are contained in the data. The $\sum R^2$ for the consistency test must be smaller than those obtained for the two-atom-type solutions. To ensure that the three distances obtained from the above steps constitute a true minimum, each distance was allowed to vary one at a time. The residuals from the consistency test were comparable to the estimated error. Total error for each ligand was determined by changing the distance to a different value while holding all other parameters constant in the fitting procedure until the $\sum R^2$ increased by a factor of 2 on each side of the minimum. A detailed discussion of error analysis is given by Powers and Kincaid (1989).

RESULTS

The optical absorption spectra of LPO native enzyme and compound III are compared in Figure 1a. Yamazaki et al. (1966) describe three methods for preparing compound III: (a) the reaction of compound II with H_2O_2 (in practice, excess H_2O_2 is added to native enzyme when the enzyme contains endogenous donors), (b) formation of ferrous compound by the addition of dithionite, then reaction with O_2, or (c) the reaction of ferric enzyme with superoxide. Previous experience has shown that the first method gave the best compound III at the concentrations needed for XAS studies. Compound III is easily distinguished from other forms by both the Soret peak and visible bands (Kohler et al., 1988). Although the expected decrease in Soret intensity of compound III was observed (Figure 1a), no bleaching of the enzyme was observed at the high concentrations of H_2O_2 used. The compound III used for XAS studies retained most of its activity after being thawed and could be converted to native enzyme.

Figure 1b shows that the ferrous form appeared to be low-spin at pH 6.0 (ferrous-434) and high-spin at pH 9.2 (ferrous-445), as reported in the case of intestinal peroxidase (Kimura et al., 1981). At pH 6, within 20 min at room temperature, the Soret peak shifted from 445 to 434 nm while the absorbance at 597 nm decreased and a new shoulder appeared at 536 nm (Figure 1b). It should be noted that the ferrous-434 form exhibited a visible spectrum different from that of either typical high- or low-spin form. At pH 9.2, however, the 445 form was stable for at least 1 h at room temperature and exhibited a typical high-spin spectrum. Storage of the concentrated samples in liquid N_2 for approximately 2 days resulted in no changes in the 434 form at pH 6.0. However, the sample at pH 9.2 converted slowly from the 445 form to the 434 form,

Table I: Results of Two-Atom Fitting and Three-Atom Consistency Test to the First Coordination Shell of Fourier Transform for Native LPO

	model	n	r (Å)	$\Delta\sigma^2$	ΔE_0	$\sum R^2$
A	Fe–N	5	2.01	1.45×10^{-3}	0.7	2.5
	Fe–N	1	2.16	-3.66×10^{-3}	−2.7	
B	Fe–N	4	2.04	1.36×10^{-3}	0.1	4.7
	Fe–N	1	1.95	5.56×10^{-3}	3.4	
C	Fe–N	4	2.05	-2.82×10^{-3}	0.7	3.3
	Fe–N	2	1.99	3.97×10^{-3}	−0.4	
G	Fe–N	4	2.036 ± 0.02	6.47×10^{-3}	0.4	0.9
	Fe–N	1	1.905 ± 0.02	9.68×10^{-3}	−1.6	
	Fe–N(O)	1	2.016 ± 0.03	5.27×10^{-3}	1.0	

Table II: Results of Two-Atom Fitting and Three-Atom Consistency Test to the First Coordination Shell of Fourier Transform for LPO Compound III

	model	n	r (Å)	$\Delta\sigma^2$	ΔE_0	$\sum R^2$
A	Fe–N	5	2.02	-2.5×10^{-3}	−4.7	3.9
	Fe–N	1	1.70	-3.3×10^{-3}	−4.2	
B	Fe–N	5	2.03	3.3×10^{-3}	−2.4	4.6
	Fe–N	1	1.93	8.5×10^{-3}	−7.9	
C	Fe–N	4	2.02	5.2×10^{-3}	−1.3	4.1
	Fe–N	2	1.96	-7.4×10^{-3}	−7.4	
D	Fe–N	5	2.02	1.7×10^{-3}	−3.6	6.3
	Fe–N	2	2.09	7.5×10^{-3}	11.1	
E	Fe–N	4	1.96	3.1×10^{-3}	−4.1	2.8
	Fe–N	1	2.10	9.6×10^{-3}	10.0	
	Fe–N(O)	1	1.70	-7.9×10^{-3}	−11.0	
F	Fe–N	4	2.02 ± 0.02	4.5×10^{-3}	−0.8	1.6
	Fe–N	1	1.91 ± 0.03	7.6×10^{-3}	7.1	
	Fe–N(O)	1	1.71 ± 0.04	-7.3×10^{-3}	0.0	

and therefore the ferrous-445 sample was prepared immediately before XAS data collection.

Chance et al. (1986c) have established a correlation between LFIR ratio and the displacement of the iron from the porphyrin plane. According to this correlation, an LFIR ratio of 1.09 for native LPO suggests that the iron is displaced by ~0.38 Å from the porphyrin plane. For compound III, with an LFIR of 1.04, and both the ferrous forms, with LFIR ratios of 1.00, this correlation suggests an iron displacement of ~0.30 and ~0.28 Å, respectively. Errors in the LFIR analysis are shown in Table IV.

The results of the first coordination shell analysis for native LPO are summarized in Table I. The $\sum R^2$ for fits with a total of six ligands are nearly a factor of 2 smaller than those with a total of five ligands. The three-atom-type consistency test further confirms that native enzyme has six ligands. Amplitude ratios of 5/1, 4/1, and 4/2 indicated that 2.16 and 1.95 Å are possible solutions for the axial ligands. Therefore, after error analysis of each bond length value, the final solution for the first coordination shell is Fe–N_p = 2.036 ± 0.015 Å, Fe–N_ϵ = 1.905 ± 0.020 Å, and Fe–N(O) = 2.16 ± 0.03 Å. We have similarly summarized the average distances of the first coordination shell of compound III in Table II and the ferrous compounds in Table III.

The fitting results for compound III (Table II) are not as well defined as those for the native enzyme. Unlike the native or the ferrous forms, three possible solutions are found for the two axial distances: 1.70, 1.92, and 2.09 Å. There are two possible explanations for this result. Either one or more of the solutions are artifacts of the fitting procedure, where two contributions are averaged as one, or multiple species, each containing different contributions, are present in the sample. The three-atom-type consistency test has been shown to distinguish fitting artifacts (Chance et al., 1983; Powers et al., 1984) from mixtures (Woolery et al., 1985). The results

Table III: Results of Two-Atom Fitting and Three-Atom Consistency Test to the First Coordination Shell of Reduced LPO

	model	n	r (Å)	$\Delta\sigma^2$	ΔE_0	$\sum R^2$
			pH 6			
A	Fe–N	5	2.03	3.14×10^{-3}	0.1	1.6
	Fe–N	1	1.93	8.23×10^{-3}	−5.0	
B	Fe–N	5	2.02	3.46×10^{-3}	−0.6	2.4
	Fe–N	1	2.18	8.31×10^{-3}	4.3	
C	Fe–N	4	2.04	4.62×10^{-3}	0.7	1.7
	Fe–N	2	1.94	6.89×10^{-3}	−2.9	
D	Fe–N	4	2.02 ± 0.02	6.20×10^{-3}	0.3	1.0
	Fe–N	1	1.91 ± 0.02	9.80×10^{-3}	−5.6	
	Fe–N(O)	1	2.18 ± 0.03	5.10×10^{-3}	−2.1	
			pH 9			
E	Fe–N	5	2.02	8.3×10^{-3}	0.1	4.2
	Fe–N	1	1.91	7.7×10^{-3}	−2.7	
F	Fe–N	4	2.02	8.4×10^{-3}	0.2	2.7
	Fe–N	1	1.90	7.8×10^{-3}	−1.7	
G	Fe–N	5	2.00	5.9×10^{-3}	−0.8	5.4
	Fe–N	1	2.38	4.5×10^{-4}	−6.4	
H	Fe–N	4	2.03 ± 0.02	8.1×10^{-3}	−6.0	1.3
	Fe–N	1	1.90 ± 0.03	7.1×10^{-3}	0.2	
	Fe–N(O)	1	2.27 ± 0.04	-7.8×10^{-3}	−6.1	

of the three-atom-type consistency tests for compound III are given in Table II (E and F). Only two combinations of the three axial solutions were found. The second (F) has physically reasonable parameters and a $\sum R^2$ which is a factor of 2 less than the first combination (E). Further, the first combination (E) has ΔE_0 parameters for two contributions which are large and in opposite directions. While this is not physically impossible, it renders this combination less reasonable than F. On the basis of previous experience with peroxidase samples (Chance et al., 1984, 1986a,b; Woolery et al., 1985; Sinclair et al., 1992), the most likely explanation of these solutions is that combination F represents a large portion of the sample (>80%) and combination E arises from a contaminating species. Various other combinations were also used to confirm these results and rule out other possibilities. Data collected at the different synchrotron sources were analyzed separately and gave identical results.

Table III shows the results for ferrous LPO at pH 6 and 9. Comparing solution D (ferrous-434, pH 6) with solution H (ferrous-445, pH 9), the only effect of changing the pH from 6 to 9 is a lengthening of one of the axial distances by ~0.2 Å.

DISCUSSION

Based on the correlation of the LFIR ratio, Fe–N_p average distance, and the Fe out-of-plane displacement (Chance et al., 1984, 1986c), these values suggest displacement of 0.33, 0.30, and 0.28 Å, respectively, for LPO native, compound III, and both ferrous forms. The LFIR ratio for native LPO is lower than for other peroxidases we have examined [HRP (Chance et al., 1984; Penner-Hahn et al., 1984, 1986), CcP (Chance et al., 1984, 1986a), and LiP (Sinclair et al., 1992)]. However, as has been observed in these other systems, the iron moves toward the heme plane in both the compound III and ferrous forms. Since the shape of the LFIR region is a consequence of multiple scattering, ruffling of the heme, for example, could bias these results.

A summary of our fitting results is shown in Figure 4. In order to understand the active-site structure of LPO, these results are compared with other spectroscopic and biochemical data for LPO as well as with XAS data for other peroxidases.

While the Fe–N_p and Fe–N_ϵ distances are similar for the different forms of the three enzymes, there are striking differences in the Fe–distal ligand distance. A distal ligand

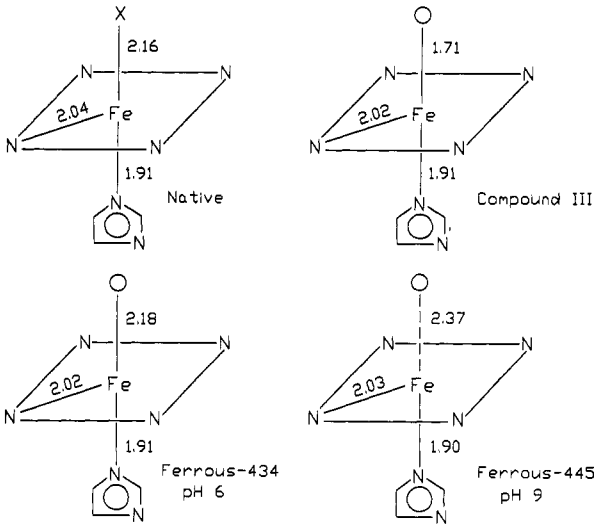

FIGURE 4: Active-site structure of LPO native, compound III, and ferrous forms. Distances are given in angstroms, and errors are given in the text. O represents a distal ligand that is likely to be an oxygen atom. X represents a distal ligand where the ligand identity is less certain.

Table IV: First Coordination Shell Distances for LPO, HRP, LiP, and Their Derivatives

	$Fe-N_p$[a] (Å)	$Fe-N_\epsilon$[b] (Å)	Fe–N(O)[c] (Å)	LFIR[d]
LPO				
native	2.04	1.91	2.16	1.09
compound III	2.02	1.91	1.71	1.04
ferrous-434, pH 6	2.02	1.91	2.18	1.00
ferrous-445, pH 9	2.03	1.90	2.37	1.00
HRP[e]				
native	2.04	1.90	2.39	1.33
compound III	2.02	1.92	1.82	1.00
ferrous	2.01	1.90	2.38	1.00
LiP H2[f]				
native	2.055	1.93	2.17	1.55
compound III	2.02	1.90	1.74	1.06
ferrous	2.02	1.91	2.10	1.07

[a] Average iron–pyrrole nitrogen distance, ±0.02 Å. [b] Iron–proximal (imidazole) nitrogen distance, ±0.02 Å. [c] Iron–distal ligand (nitrogen or oxygen) distance, ±0.03 Å. [d] Ligand field indicator region parameter in the ratio of absorbance peaks at 7175 and 7195 eV as defined in text. LFIR error ±0.05. [e] Chance et al. (1984). [f] Sinclair et al. (1992).

at <2.2 Å would result in a six-coordinate heme iron. However, a distal ligand at ~2.4 Å would not be directly coordinated and would result in a five-coordinate heme iron. As shown in Table IV, the results for native LPO and compound III are strikingly similar to those for LiP (Sinclair et al., 1992) and significantly different from those for HRP (Chance et al., 1984). Native forms of LPO and LiP are both six-coordinate at ~−100 °C and have identical axial ligand distances within the error. The sixth ligand in each case is suggested to be H_2O (Sievers, 1980; Sievers et al., 1983; Kitagawa et al., 1983; Manthey et al., 1986; Hashimoto et al., 1989; Andersson et al., 1987). The distance to the sixth ligand, Fe–O, is 2.17 Å for LPO and LiP but 2.40 Å for the five-coordinate HRP. While all compound III structures are six-coordinate, the structures of LPO and LiP compound III are identical within the error but have an Fe–O axial distance that is ~0.1 Å shorter than that of HRP. The six-coordinate ferrous form of LPO (ferrous-434) is very similar to that of LiP while the five-coordinate ferrous form (ferrous-445) is identical within the error to that of HRP.

Resonance Raman studies indicate that native LPO remains six-coordinate at room temperature. The Fe–O (presumably from H_2O) in LPO is nearly 0.2 Å shorter than that in HRP, a fact that supports the suggestion of Sievers (1980) that LPO has a narrow heme pocket. This places the heme iron and the sixth ligand closer to distal residues and may partially explain the stronger hydrogen bonding between the sixth ligand and distal bases observed in LPO compound II by Reczek et al. (1989). These residues have been shown to play a major role in the heterolytic cleavage of peroxide in CcP (Poulos & Kraut, 1980), and their closeness to the iron heme may facilitate the formation of compound I in LPO (Ohlsson, 1984). The fact that the Fe–O bond distance in compound III is somewhat shorter for LPO than for HRP further supports the idea that LPO may have a narrow heme pocket. These differences might also be a factor in the unique reactivity of LPO and LiP.

Hu and Kincaid (1991) found that the $Fe-O_2$ stretching frequency in LPO compound III was considerably lower than those reported for related proteins, suggesting that the $Fe-O_2$ bonding is considerably weaker. Although we do not find a longer Fe–O bond distance, our results are not sensitive to the Fe–O–O angle and other factors such as distal hydrogen bonding, which could lower the vibration frequency. We observe an average Fe–O distance, a value typical of oxygen bound to ferrous heme proteins.

Table IV indicates that there are two structurally different forms of ferrous LPO. The ferrous-445 form, which is relatively stable at pH 9, is five-coordinated, while the ferrous-434 form, which is produced from the ferrous-445 form at pH 6, is six-coordinated. Although the ferrous-445 form has six ligands listed in Table IV, it is unlikely that an oxygen at 2.37 Å would be directly coordinated to the iron. The six-coordinate ferrous-434 form shows an Fe–distal ligand distance that is ~0.2 Å shorter than the H_2O ligand in ferrous-445 form. However, the five-coordinate form, ferrous 445, is identical within the error to that of ferrous HRP. The distal ligand has therefore moved ~0.2 Å away from the heme iron at pH 9 when compared to pH 6. As LPO is believed to have a narrow heme pocket, this raises the possibility of rearrangement of the distal residues. If so, we see no evidence for it in the higher shell contributions, as these are identical for both ferrous forms (Figure 3). However, the lack of changes in higher order shells does not rule out the possibility of distal residue movement, as the contributions from distal amino acids are generally not observed for heme proteins. The other distances ($Fe-N_p$ and $Fe-N_\epsilon$) are unaffected by the pH change.

Although these results unambiguously show a change in the coordination number of ferrous LPO with decreased pH, they do not necessarily support an accompanying change in spin state. Chance et al. (1984) established a correlation between the magnetic moment, LFIR, and average $Fe-N_p$ distance for peroxidases and other heme proteins. According to their results, the ferrous forms are probably high or intermediate spin. For a full spin conversion in the ferrous heme protein, the average change in $Fe-N_p$ distance is expected to be at least 0.03 Å. This difference, which is greater than our estimated error, is not observed. Further, contradictory conclusions have been derived from resonance Raman studies. Manthey et al. (1986) pointed out that their results do not unambiguously support two different spin states for the ferrous forms of LPO, despite the change in the vibrational frequencies of the spin-state marker band, because the low-frequency vibrations indicate no significant differences in the character of the $Fe-N_\epsilon$ bond. The resonance Raman data obtained by Kimura et al. (1981), however, have shown that the ferrous-445 form is high-spin and the ferrous-434 form is low-spin in the case of IPO, which, similarly to LPO, exhibits a pH-

dependent change in the optical spectra of the ferrous form. There is little doubt that the ferrous-445 form of LPO is five-coordinated high-spin, as is the case with HRP and deoxymyoglobin. The ambiguous conclusions of EXAFS and resonance Raman data on the spin state of the ferrous-434 form might be related to the fact that its visible spectrum does not resemble that of a typical high- or low-spin heme.

The possible identity of the sixth ligand of the LPO ferrous-434 form can also be addressed by our data. From those that have been suggested, histidine or tyrosine ligands, which contain a rigid ring structure, are not likely. Contributions from distal amino acids which are not directly coordinated to the iron are generally not observed for heme proteins. However, those which contain a rigid ring structure have been shown to have characteristic outer-shell contributions when directly coordinated to the iron (Brown et al., 1980; Co et al., 1981; Woolery et al., 1984; Tang et al., 1992). In fact, the outer-shell contributions of both ferrous forms of LPO (Figure 3) are nearly identical within the error. Thus we are left with the possibilities of a coordinated water molecule or carboxylate group. Either of these is consistent with our results since either could produce a high or intermediate spin state with little or no contribution to the higher shells. However, both of these possibilities raise difficult questions. Current results indicate that, at pH 6, the ferrous form is five-coordinate (like that at pH 9, ferrous-445) at early times and rapidly converts to a six-coordinate ferrous-434 form. Raising the pH to 9 is believed to stabilize the five-coordinate form since conversion to the six-coordinate form is much slower. If the sixth ligand in the ferrous-434 form at pH 6 is water, why does the enzyme immediately produce a five-coordinate ferrous species at pH 6 and 9 and then rebind the water that was already present as a sixth ligand in the native state at a pH-dependent rate? Alternately, if the sixth ligand in the ferrous-434 form at pH 6 is a carboxylate group, it would have to be deprotonated to bind the ferrous iron heme. Why does increasing the pH prevent this binding? Clearly, further work is required to address these questions.

ACKNOWLEDGMENT

We thank the National Bio-structures Participating Research Team for allocation of beam time and for experimental support on X-9 at the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY. We also thank the staff of the Stanford Synchrotron Light Source, Palo Alto, CA, for beam time and experimental support where part of this work was performed under proposal 2149 BP.

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