Distinct Heme Active-Site Structure in Lactoperoxidase Revealed by Resonance Raman Spectroscopy†

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ABSTRACT: Low-frequency resonance Raman spectra of the cyanide and carbon monoxide adducts of lactoperoxidase are obtained with Soret excitation. The ν(Fe–CN) and δ(Fe–C–N) modes are detected at 360 and 453 cm^{-1}, respectively. Upon the isotopic substitution of $^{13}C^{14}N$, $^{12}C^{15}N$, and $^{13}C^{15}N$, the band at 453 cm^{-1} in the natural abundance adduct shifts to 448, 452, and 445 cm^{-1}, while the 360-cm^{-1} peak shifts to 358, 357, and 356 cm^{-1}, respectively. The 360-cm^{-1} band is shifted to 355 cm^{-1} when the pH is changed from 7.0 to 10.5. On the basis of a previous normal-mode analysis of the cyanoferric adduct of myeloperoxidase, a bent Fe–C–N linkage is suggested for the cyanide adduct of lactoperoxidase. The ν(Fe–CN) (374 cm^{-1}) and δ(Fe–C–N) (480 cm^{-1}) modes are observed for the cyanide adduct of reduced lactoperoxidase. For the carbon monoxide adduct, the ν(Fe–CO) (533 cm^{-1}) and δ(Fe–C–O) (578 cm^{-1}) modes at pH 7.0 are observed to shift to 498 and 570 cm^{-1} as the pH is raised from 7.0 to 10.0. The strong intensity of δ(Fe–C–O) at both acid and alkaline pHs, along with a suggested bent structure of the Fe–C–N moiety, implies a narrow heme pocket for lactoperoxidase.

Lactoperoxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7), isolated from milk, saliva and tears, catalyzes the peroxidation of endogenous thiocyanate in the presence of hydrogen peroxide (Pruit, 1985; Reiter & Perraudin, 1991). The resulting antimicrobial hypothiocyanite makes the enzyme an essential component of the biological defense system of mammals. The active site of the enzyme involves a heme group that is extraordinarily tightly bound to a single peptide of a molecular weight 78 000, of which about 10% is carbohydrate (Carlstrom, 1969; Sievers, 1981). The heme group has recently been identified to be a modified derivative of protoporphyrin IX, whose 8-methyl substituent is replaced by an 8-mercaptomethyl group and linked to apoprotein through the formation of a disulfide bond (Nichol et al., 1987). Like many other peroxidases, the enzyme reacts with hydroperoxides to form a primary intermediate, compound I, which rapidly decays to compound II in the absence of reducing agent (Kimura & Yamazaki, 1979). Further reaction of compound II with excess hydrogen peroxide leads to the formation of compound III, which can also be formed either by the oxygenation of the ferrous enzyme (Hu & Kincaid, 1991) or by the reaction of the ferric enzyme with enzymatically generated superoxide (Metodiewa & Dunford, 1989).

Extensive spectroscopic studies of lactoperoxidase, including MCD (Sievers et al., 1983), EPR (Sievers et al., 1984; Lukat et al., 1987), NMR (Behere et al., 1985a,b; Goff et al., 1985; Shiro & Morishima, 1986; Thanabal & La Mar, 1989), and resonance Raman (RR) (Kitagawa et al., 1983; Manthey et al., 1986; Hashimoto et al., 1989), have established that the enzyme contains a five-coordinate high-spin ferric heme in the resting state with histidine serving as the proximal ligand. While the amino acid sequence of the apoenzyme was recently determined (Cals et al., 1991), the three-dimensional structure is not yet available. Information regarding the active-site environment is also scarce. We recently reported the resonance Raman characterization of lactoperoxidase compound III (Hu & Kincaid, 1991). The observation of an unusually low ν(Fe–O_2) frequency (531 cm^{-1}), along with the detection of a similarly low ν(Fe=O) frequency (745 cm^{-1}) for compound II (Reczek et al., 1989), prompted us to conduct further RR spectral studies of the active-site structure of derivatives of this enzyme with the diatomic exogenous ligands carbon monoxide and cyanide. These ligands have been proven to be very useful RR probes for the heme active-site structure (Yu, 1986; Kerr & Yu, 1988). Analysis of the low-frequency iron–ligand vibrations, including ν(Fe–CO), δ(Fe–C–O), ν(Fe–CN), and δ(Fe–C–N) modes, allows us to compare lactoperoxidase with structurally well-defined peroxidases such as cytochrome *c* peroxidase (Poulos & Kraut, 1980; Finzel et al., 1984) and myeloperoxidase (Zheng & Fenna, 1992).

EXPERIMENTAL PROCEDURES

Materials. Lactoperoxidase was isolated and purified from unpasteurized cow milk, according to the published procedures (Morrison, 1970; Morrison & Hultquist, 1963). The purity index, R_z (i.e., A_{413}/A_{280}), value of the enzyme preparation used in this work was 0.91 or greater. Concentrated solutions of samples (about 1 mM) were stored under liquid nitrogen until ready for use. The concentration was measured spectrophotometrically using ϵ = 112 $mM^{-1}\cdot cm^{-1}$ at 413 nm. $K^{12}C^{15}N$ (99%) and $K^{13}C^{15}N$ (both 99%) were purchased from Isotech (OH). $K^{13}C^{14}N$ (99%) was from Aldrich (Milwaukee, WI) and $^{13}C^{16}O$ from ICON Services (Summit, NJ).

Spectroscopic Measurements. Resonance Raman spectra were acquired on a Spex Model 1403 system equipped with a Spex DM1B data station and a Hamamatsu R-928 photomultiplier. Excitation lines were from Coherent Innova Model 100-K3 Kr^+ ion (413.1 nm) and Linconix Model 4240NB helium:cadmium (441.6 nm) lasers. The NMR tube containing enzyme solutions was positioned in a backscattering geometry and was kept spinning to lessen photodissociation and local heating at room temperature throughout the spectral acquisition.

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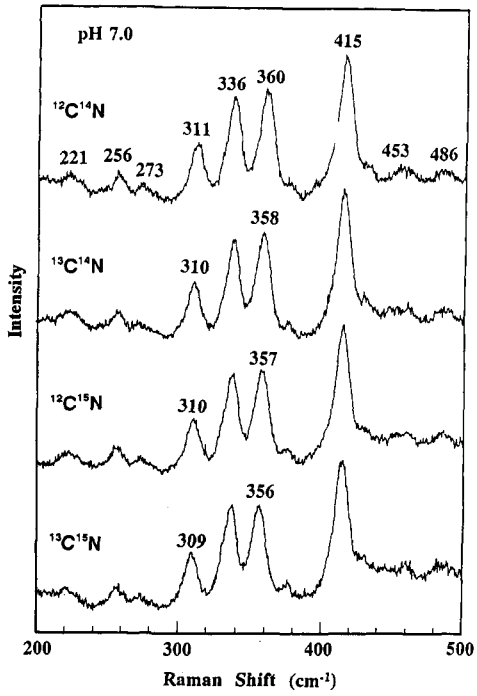

FIGURE 1: Low-frequency resonance Raman spectra of the cyanide adduct of ferric lactoperoxidase in 100 mM potassium phosphate (pH 7.0) buffer. The enzyme concentration was 100 μM. Excitation line, 413.1 nm; laser power at sample point: 20 mW; spectral slits, 6 cm^{-1}; 0.5 cm^{-1}/0.5 s; 5 scans.

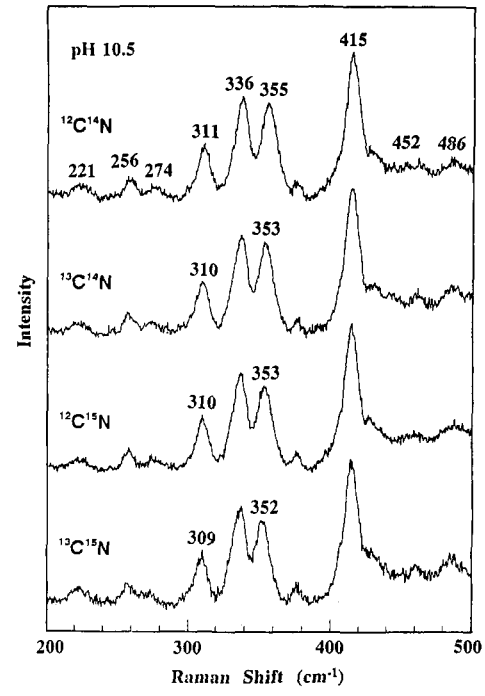

FIGURE 2: Low-frequency resonance Raman spectra of the cyanide adduct of ferric lactoperoxidase in 100 mM sodium carbonate (pH 10.5) buffer. Other conditions are the same as those in Figure 1.

RESULTS

Cyanide Adduct of Ferric Lactoperoxidase. Figure 1 shows the low-frequency resonance Raman spectra (200–500 cm^{-1}) of the cyanide adducts of ferric lactoperoxidase, along with those of the $^{13}C^{14}N$, $^{12}C^{15}N$, and $^{13}C^{15}N$ analogues to effectively identify those modes associated with the Fe(III)–CN linkage. It is immediately apparent that the 360-cm^{-1} band exhibits a monotonic downshift as the mass of cyanide increases from $^{12}C^{14}N$ (360 cm^{-1}), through $^{13}C^{15}N$ (358 cm^{-1}) and $^{12}C^{15}N$ (357 cm^{-1}), to $^{13}C^{15}N$ (356 cm^{-1}). Therefore, this band is assigned to the ν(Fe–CN) mode. At alkaline pH, the ν(Fe–CN) mode is shifted from 360 to 355 cm^{-1} Figure 2. Another line at 311 cm^{-1} exhibits a small, but reproducible, isotopic shift in the spectrum of the $^{13}C^{15}N$ adduct at both pHs (Figures 1 and 2). In addition, intensity alterations were noticed for a broad spectral feature at about 450 cm^{-1}. To unambiguously establish the isotope shifts of this weak feature, spectra in this region were acquired with much higher resolution (4-cm^{-1} spectral slits) and signal-to-noise ratio (Figure 3). The band at 453 cm^{-1} in the natural-abundance adduct shifts to 448, 452, and 445 cm^{-1} upon the isotopic substitution of $^{13}C^{14}N$, $^{12}C^{15}N$, and $^{13}C^{15}N$. This zigzag pattern, being characteristic of a bending mode (Tsubaki et al., 1982; Yu et al., 1984), suggests this band is in the δ(Fe–C–N) mode. At alkaline pH, the δ(Fe–C–N) mode shows small changes in frequency and isotopic shift.

Cyanide Adduct of Ferrous Lactoperoxidase. Ferrous lactoperoxidase binds cyanide to form a six-coordinate, low-spin complex (Manthey et al., 1986). The Soret band of this complex maximizes at 435 nm (Carlstrom, 1969); thus the 441.6-nm line of the He:Cd laser was used to obtain the RR spectrum with the B-state resonance. Figure 4 shows the low-frequency RR spectra of the cyanide adduct of ferrous lactoperoxidase. The strong band at 374 cm^{-1}, showing a monotonic downshift, is assigned to the ν(Fe–CN) mode (Han et al., 1989). A weak and broad band at 480 cm^{-1} shifts to

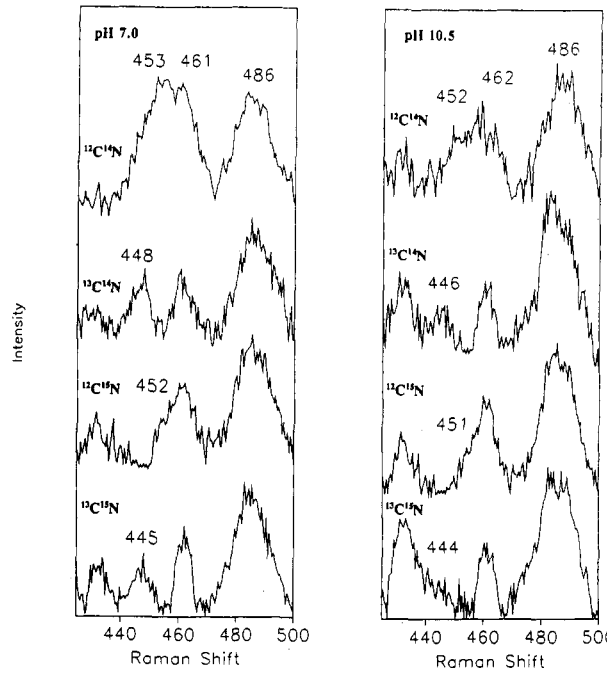

FIGURE 3: Low-frequency resonance Raman spectra of the cyanide adduct of ferric lactoperoxidase at pH 7.0 (left panel) and pH 10.5 (right panel), showing the identification and isotope shifts of the δ(Fe–C–N) mode. Protein concentrations and buffers are the same as in Figures 1 and 2. Spectral slit: 4 cm^{-1}; 0.5 cm^{-1}/2 s; 55 scans for each trace.

473 ($^{13}C^{14}N$), 477 ($^{12}C^{15}N$), and 472 ($^{13}C^{15}N$) cm^{-1} as the mass of the bound cyanide increases; the frequencies of these broad bands correspond to the center of the peak. It is therefore assigned to the δ(Fe–C–N) mode. A band at 315 cm^{-1} displays a 2-cm^{-1} shift in the spectrum of the $^{13}C^{15}N$ adduct, analogous to the spectral feature at 311 cm^{-1} in Figure 1. Comparison of Figures 1 and 4 shows that addition of one electron into the cyanide adduct of ferric lactoperoxidase leads to frequency shifts of some heme modes. Thus, the 221-, 256-, 273-, 311-,

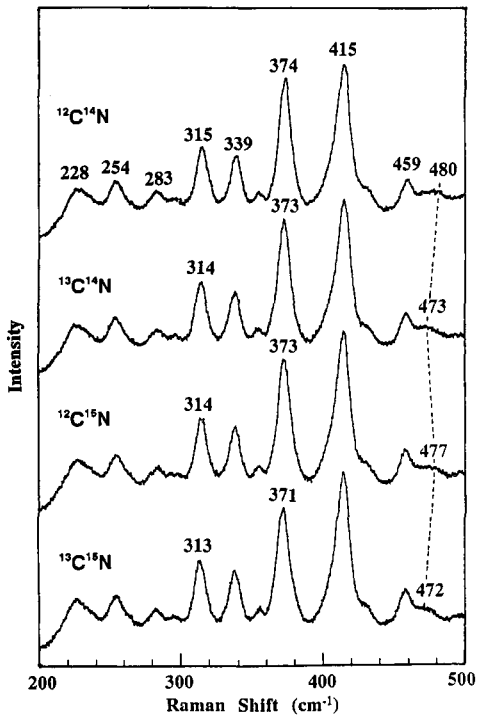

FIGURE 4: Low-frequency resonance Raman spectra of the cyanide adduct of ferrous lactoperoxidase in 100 mM potassium phosphate (pH 7.0) buffer. The enzyme concentration was 100 μM. Excitation line, 441.6 nm, laser power at sample point, 15 mw; 0.5 cm^{-1}/0.5 s; 5 scans.

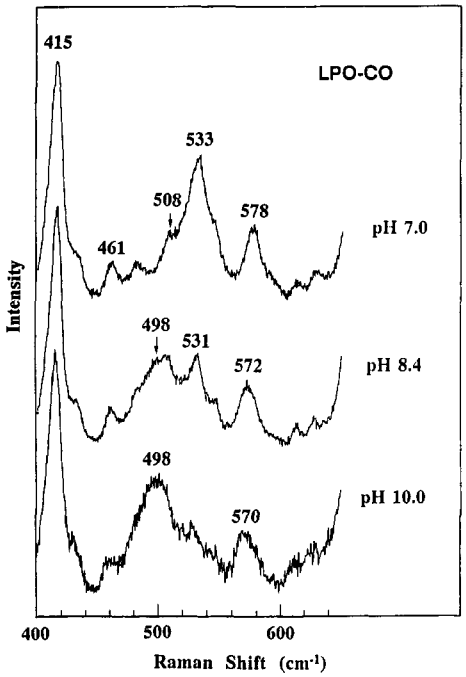

FIGURE 5: Low-frequency resonance Raman spectra of the natural-abundance CO adduct of lactoperoxidase in different pHs. Buffers: pH 7.0: 100 mM potassium phosphate; pH 8.5, 100 mM Tris-HCl; pH 10, 100 mM sodium bicarbonate. Excitation line, 413.1 nm. Laser power was 5 mW for the sample at pH 7.0 and 1 mW for samples at pH 8.4 and 10.0. Spectral accumulation, 0.5 cm^{-1}/0.5 s; 10 scans.

and 336-cm^{-1} bands in Figure 1 are observed at 228, 254, 283, 315, and 339 cm^{-1}.

Carbon Monoxide Adduct of Ferrous Lactoperoxidase. Figure 5 displays the RR spectra of the carbon monoxide adduct of lactoperoxidase at pH 7.0, 8.4, and 10.0. Figure 6 shows the effect of ^{13}CO isotopic substitution on the RR spectra under the same conditions as those indicated in Figure

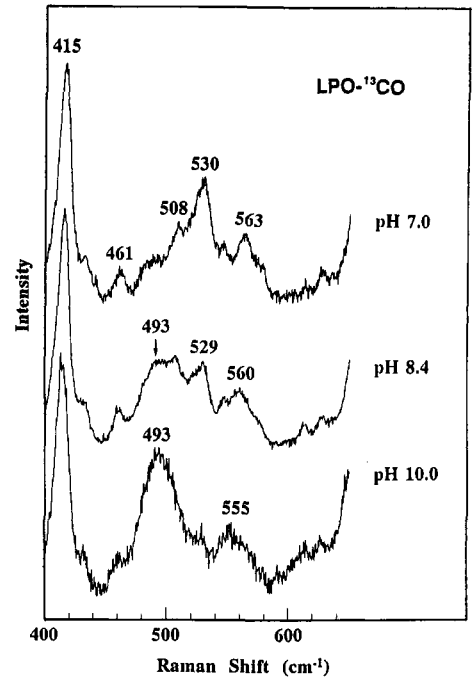

FIGURE 6: Low-frequency resonance Raman spectra of the ^{13}CO adduct of lactoperoxidase at different pHs. Experimental conditions are the same as those described in the legend of Figure 5.

5. The spectra in both figures are normalized using the 415-cm^{-1} band as an internal intensity standard. At pH 7.0, two bands are clearly associated with the Fe–CO linkage based on their obvious isotopic shifts. The band at 533 cm^{-1} is in the region wherein ν(Fe–CO) frequencies for several heme proteins are normally observed and show a typical isotopic shift pattern. Another band at 578 cm^{-1} exhibits a 15-cm^{-1} downshift in the spectrum of the ^{13}CO adduct, which is characteristic of the δ(Fe–C–O) vibration (Tsubaki et al., 1982). Therefore, these two bands are assigned to ν(Fe–CO) and δ(Fe–C–O) vibrations, respectively.

When the pH is raised from 7.0 to 10.0, the ν(Fe–CO) mode moves to 498 cm^{-1}, while the δ(Fe–C–O) shifts from 578 to 570 cm^{-1}. At an intermediate pH (8.4), both bands assignable to ν(Fe–CO) mode are observed with comparable intensities. At the same time, the δ(Fe–C–O) mode, located at 572 cm^{-1}, becomes broader. The assignments of these bands are secured from their ^{13}CO isotopic shift, shown in Figure 6.

DISCUSSION

Structure of the Fe–CN Linkage in Cyanoferric Lactoperoxidase. RR and IR spectroscopy has been previously used to investigate the three normal modes of the Fe–CN linkage: RR for the enhancement of ν(Fe–CN) and δ(Fe–C–N) (Yu et al., 1984; Henry et al., 1985; Tanaka et al., 1987; Uno et al., 1988; Han et al., 1989; Lopez-Garriga et al., 1990; Surerus et al., 1992) and IR for the detection of ν(C–N) (Yoshikawa et al., 1985). Although only ν(Fe–CN) has been detected for model compounds (Tanaka et al., 1987), two distinctly different RR spectral patterns are documented for the CN^- adducts of heme proteins. Yu and co-workers (Yu et al., 1984) assigned two isotope-sensitive lines in the spectra of cyanomet monomeric CTT hemoglobin III at 453 and 412 cm^{-1} to the ν(Fe–CN) and δ(Fe–C–N) modes, respectively, based upon isotope shifts patterns. The ν(Fe–CN) band is also detected for the cyanide adduct of methemoglobin and myoglobin (Henry et al., 1985). In these

cases, ν(Fe–CN) lies at higher frequency than δ(Fe–C–N). However, in the RR spectra of the cyanide adduct of sulfite reductase, Han et al. (1989) observed four isotope-sensitive lines and associated a pair of bands that show a reversed order of isotopic dependence, i.e., the higher frequency one (451 cm^{-1}) with a zigzag isotopic shift and a steadily decreasing 352-cm^{-1} band. A more clear-cut example is seen in the spectra of cyanoferric myeloperoxidase (Lopez-Garriga et al., 1990).

Our observation of the zigzag (453 cm^{-1}) and monotonic (365 cm^{-1}) isotope-shift bands for the cyanide adduct of ferric lactoperoxidase suggests the Fe–CN linkage is similar to that of myeloperoxidase. In the normal-mode analysis carried out by Lopez-Garriga et al. (1990) and Han et al. (1989), the observed frequency and isotope shift can only be explained by using a bent Fe–C–N geometry, which surprisingly is different from the linear Fe–CN linkage seen in the cyanide adduct of iron porphyrin (Scheidt et al., 1983). However, the band assignments of these two calculations seem contradictory. While Han et al. (1989) assigned the bands at about 450 and 350 cm^{-1} to the ν(Fe–CN) and the δ(Fe–C–N) mode, respectively, the opposite assignment was clearly supported by the calculation of Lopez-Garriga (1990). The difference may be attributable to the use of the more simplified model by Han et al. (1989), and the latter treatment which assigned the 365-cm^{-1} band to the ν(Fe–CN) mode is consistent with its much higher intensity [relative to that of δ(Fe–C–N) at 453 cm^{-1}].

In the cyanide adduct of ferrous lactoperoxidase, the Fe–CN moiety is isoelectronic with the CO derivative; thus, a linear Fe–C–N linkage is expected in the absence of steric distortion. Han et al. (1989) detected the ν(Fe–CN) (452 cm^{-1}) and δ(Fe–C–N) (495 cm^{-1}) modes for the cyanoferrous sulfite reductase. The mode characteristics are similar to those of the CO adduct, but their frequencies are much lower, owing to reduced π-back bonding. Interestingly, the ν(Fe–CN) of lactoperoxidase is observed at a significantly lower frequency (374 cm^{-1}), while the δ(Fe–C–N) mode (480 cm^{-1}) is nearly the same as that of sulfite reductase (495 cm^{-1}). The large frequency spreading of the ν(Fe–CN) and δ(Fe–C–N) modes in lactoperoxidase may be caused by a bent structure of the Fe–C–N linkage, which tends to separate these two modes through a kinematic effect (Hu & Kincaid, 1991). This large frequency variation suggests that the ν(Fe–CN) mode of the CN adduct of reduced heme proteins (374–452 cm^{-1}) may be an effective probe of the heme active-site structure.

Manthey et al. (1986) previously studied the RR spectra of cyanoferrous lactoperoxidase and noticed a time-dependent change of several low-frequency features, particularly the bands at 254 and 374 cm^{-1}. Their suggestion that the 374-cm^{-1} band corresponds to ν(Fe–His) apparently differs from our assignment of this mode to the ν(Fe–CN) mode, which is supported by the cyanide isotope shift. It seems reasonable to attribute the previously observed spectral change to partial oxidation of the reduced adduct, noting that close examination of their spectra reveals that the bands at 226, 339, and 374 cm^{-1} are broadened. As noted under Results, these bands have distinct frequencies in the cyanide adduct of ferric and reduced lactoperoxidase.

In conclusion of this section, some comments are warranted regarding the spectral features at 311 and 315 cm^{-1} for the cyanide adduct of ferric and ferrous lactoperoxidase, respectively. Although Yu and co-workers (Kerr & Yu, 1984; Gersonde et al., 1986) have assigned a similar band to the ν(Fe–His) vibration in the RR spectra of the ferric CN^- and ferrous CO adducts, we have argued (Hu & Kincaid, 1991a) elsewhere that this mode is more likely an out-of-plane vibration. The resulting shift upon cyanide isotope substitution can be explained by coupling to the Fe–CN vibrations, which are also out-of-plane in nature.

Heme Active-Site Environment. The pH-induced frequency change of the ν(Fe–CO) mode in lactoperoxidase is quite similar to those reported for the CO adducts of horseradish peroxidase (Evangelista-Kirkup et al., 1986; Uno et al., 1987) and cytochrome *c* peroxidase (Smulevich et al., 1986). In these two proteins, the very strong Fe–CO bond evident at neutral pH was explained by the formation of distal hydrogen bonding, which enhances π-back bonding. The high-resolution X-ray crystal structure of cytochrome *c* peroxidase (Poulos & Kraut, 1980) clearly shows the presence of a distal histidine. Raising the pH above the pK_a value ($\sim$8.4) titrates the proton of the distal histidylimidazole, thus eliminating the hydrogen bonding and lowering the ν(Fe–CO). It is therefore plausible that a similar interaction occurs in the heme pocket of lactoperoxidase. The similarities of both the magnitude of the ν(Fe–CO) frequency shift and the pK_a value suggest a structural homology near the heme site among these peroxidases from various sources (plant, bacteria, and mammalian).

While these similarities are observed, there are also subtle differences present in the RR spectra. We call attention to the intensity of the δ(Fe–C–O) mode and its pH-dependence. This mode is frequently observed in the RR spectra of heme proteins (Tsubaki et al., 1982) and a few strapped heme models (Yu et al., 1983), but not in those of many other model analogues without distal distortion. It has been proposed that the enhancement of this mode is indicative of an off-axis displacement of the Fe–C–O linkage and that the intensity measures the degree of distortion (Yu et al., 1983). A particularly interesting observation is the strong enhancement of the δ(Fe–C–O) mode at 570 cm^{-1} in the RR spectrum of the lactoperoxidase CO adduct at alkaline pH. This is in sharp contrast to the situation experienced in horseradish peroxidase (Evangelista-Kirkup et al., 1986) and cytochrome *c* peroxidase (Smulevich et al., 1986), for which a weak δ(Fe–C–O) band at acid pH becomes essentially undetectable at alkaline pH. Two binding modes of the Fe–C–O moiety are suggested to account for the frequency changes and enhancement patterns of the observed Fe–CO vibrations. At acid pH, the Fe–C–O linkage forms a hydrogen bond with the distal histidine and is tilted, relative to the heme plane. This form is then converted to an upright geometry, perpendicular to the heme plane, with concomitant loss of hydrogen bonding. The strong intensity enhancement of the δ(Fe–C–O) mode for lactoperoxidase at both acid and alkaline pH suggests that the Fe–C–O linkage is significantly distorted, likely in a tilted fashion. Removal of hydrogen bonding does not relieve this distortion, although the frequency of the δ(Fe–C–O) mode is shifted from 578 to 570 cm^{-1} as the pH is changed from 7.0 to 10.0. Such a result could arise from distortion of the bound carbon monoxide, presumably imposed by the steric bulk of nearby protein residues. This result, along with a bent Fe–C–N linkage, leads us to conclude that the heme pocket of lactoperoxidase is more constrained than those of horseradish peroxidase and cytochrome *c* peroxidase. Our conclusion is supported by recent extended X-ray absorption fine structure studies of several derivatives of lactoperoxidase (Chang et al., 1993), which appeared during the revision of this paper.

The unique heme pocket of lactoperoxidase is also revealed by the correlation of the ν(C–O) and ν(Fe–CO) frequencies.

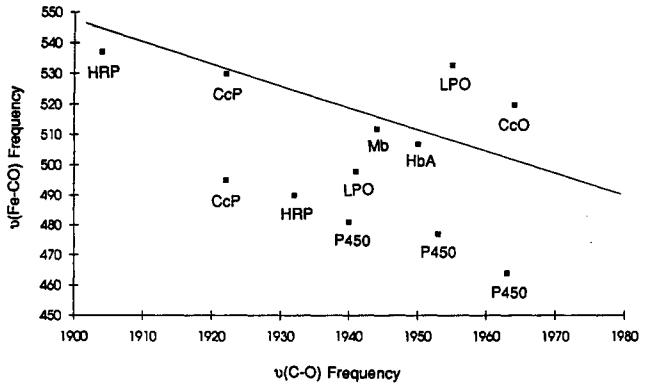

FIGURE 7: Inverse correlation relationship between the frequencies of the ν(Fe–CO) and the ν(C–O) modes. The solid line represents the regression equation $\nu_{Fe-CO} = 1935 - 0.73\nu_{C-O}$ derived from data for many CO adducts with nitrogenous proximal ligands. The scattered points are for several heme proteins. HRP, horseradish peroxidase; CcP, cytochrome *c* peroxidase; CcO, cytochrome *c* oxidase; Mb, sperm whale myoglobin; HbA, human hemoglogin A; P450:

It has been empirically established that there is an inverse correlation between the frequencies of the ν(Fe–CO) and ν(C–O) modes (Yu et al., 1983; Tsubaki et al., 1986; Uno et al., 1987; Kerr & Yu, 1988; Morikis et al., 1989; Nagai et al., 1991), attributable to changes of the Fe→CO π-back bonding, when the proximal ligands are histidine (or imidazole). Large deviations from this linear correlation are observed only for the CO adducts possessing strong electron donor groups (cytochromes P450) or weak electron donor groups (cytochrome *c* oxidase and tetrahydrofuran) as the proximal ligands. Although our attempts to detect the ν(C–O) mode of the bound CO were complicated by the high background in the expected region of 1900–2000 cm^{-1}, Smith et al. (1984) have used IR spectroscopy to detect the ν(Fe–CO) frequencies at 1941 and 1955 cm^{-1} for the CO adduct in deuterated buffer at pH 7.0. These two bands are attributed to the hydrogen-bonded (1955 cm^{-1}) and unperturbed (1941 cm^{-1}) species (Smith et al., 1984). We therefore associate the 1955- and 1941-cm^{-1} bands with the 533- and 498-cm^{-1} bands, respectively. Figure 7 shows the correlation of the RR ν(Fe–CO) and IR ν(C–O) frequencies of the CO adduct of lactoperoxidase, along with those CO adducts of several other heme proteins. The solid line corresponds to the regression equation for a large number of CO adducts of heme proteins and model compounds which have nitrogenous axial ligands in the trans position (Li & Spiro, 1988). For the CO adduct of lactoperoxidase at neutral pH, the point clearly lies above the line. This is in sharp contrast to all the neutral CO adducts of heme proteins (except cytochrome *c* oxidase) possessing histidine as the axial ligand. This result suggests a proximal histidine of much weaker donor ability. As the pH is raised to 10.0, the point falls below the line, analogous to the situation encountered for horseradish peroxidase and cytochrome *c* peroxidase. This increased electron-donation ability of the proximal histidine for alkaline peroxidases has been ascribed to the deprotonation of the coordinated imidazole group. It is rather difficult to rationalize why the electronic structure of iron–histidine for the CO adduct of lactoperoxidase is similar to those of other peroxidases at alkaline pH, but dramatically different at neutral pH. A plausible explanation is that the protein undergoes conformational changes during the acid–alkaline transition, evidence for which has previously been seen for the ferrous derivative (Carlstrom, 1969; Manthey et al., 1985). Further work is needed to evaluate how the protein conformation triggers this unique change in the bonding of iron–proximal ligand observed for lactoperoxidase.

In summary, employing resonance Raman spectroscopy to study the low-frequency Fe–CN and Fe–CO vibrations, we have observed close structural similarity of the heme active site of lactoperoxidase to those of well-characterized cytochrome *c* peroxidase and myeloperoxidase. The subtle differences observed are attributed to a more constrained heme pocket of lactoperoxidase.

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