Table III. Atomic Parameters Used in the Calculation

atom	orbital	H_{ii}, eV	ζ
Ne	2s	-40.96	2.879
	2p	-21.56	2.879
Ar	3s	-27.63	2.585
	3p	-15.76	2.255
Kr	4s	-24.36	2.829
	4p	-13.99	2.442
Xe	5s	-21.21	2.844
	5p	-12.13	2.485
F	2s	-40.0	2.425
	2p	-18.1	2.425
Cl	3s	-26.3	2.183
	3p	-14.2	1.733
Br	4s	-22.07	2.588
	4p	-13.1	2.131
I	5s	-18.0	2.679
	5p	-12.7	2.322

may eventually allow a structural determination by microwave spectroscopy.

Conclusion

In the complete Walsh diagram (Figure 3), we find three minima along a computed reaction pathway from a van der Waals complex to a linear molecule. While the extended Hückel method cannot be used to calculate the heights of the barriers between the three minima, the orbital analysis along the pathway is unambiguous. The presence of two HOMO-LUMO crossings produces two energy barriers, which are expected to be important when Ng and X_2 p-orbital energies are similar, especially for $KrCl_2$ and XeI_2. There is no way that the system can avoid these level crossings.

We believe there will be three possible geometries for $XeCl_2$ and several other NgX_2 species: the van der Waals complex, one intermediate bent state, and the linear molecule. The intermediate state is a surprise and should be the object of an experimental search. We have discussed possible experimental methods to observe both the intermediate geometry and the linear molecule in the gas phase.

Acknowledgment. We thank the Consiglio Nazionale delle Ricerche (CNR, Italy) for the award of a postdoctoral fellowship to D.M.P. and the National Science Foundation for its support through Grant CHE-8912070. We also thank Prof. P. L. Houston for a helpful discussion.

Appendix

For the computations, we use the extended Hückel method,[16] a semiempirical molecular orbital procedure, with weighted H_{ij}'s.[17] The parameters used in the calculations are reported in Table III. The Slater exponents for the noble gases are from ref 18, and the H_{ii} values, from ref 19. The three-dimensional graphics have been carried out by the computer program CACAO, described elsewhere.[20]

Registry No. $XeCl_2$, 13780-38-6; Xe, 7440-63-3; Cl, 7782-50-5.

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Resonance Raman Structural Characterization and the Mechanism of Formation of Lactoperoxidase Compound III

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Abstract: Lactoperoxidase compound III (LPO-III) is characterized by resonance Raman spectroscopy with both the Soret and Q-band excitations. The identical spectral patterns observed for LPO-III prepared by the addition of 100-fold hydrogen peroxide to ferric enzyme and by the oxygenation of the ferrous enzyme confirm the formulation of LPO-III as a low-spin dioxygen adduct. The Fe–O_2 stretching vibration (identified at 531 cm^{-1} for the $^{16}O_2$ adduct and 513 cm^{-1} for the $^{18}O_2$ derivative) is the lowest yet reported for an oxygenated heme protein. The anomalous isotopic shift of the ν(Fe–O_2) mode is attributed to vibrational coupling of the Fe–$^{18}O_2$ stretching mode with a heme mode located at 508 cm^{-1}. In addition, the Fe–O–O bending vibration is also observed at 491 cm^{-1}. The study of the ν(Fe–O_2) mode of LPO-III, prepared by different combinations of isotopically labeled peroxides, establishes unambiguously that the ferryl oxygen atom of LPO-II is displaced and the dioxygen fragment of LPO-III is derived from the hydrogen peroxide.

Introduction

Lactoperoxidase, present in mammalian milk, saliva, and tears, is involved in bacterial defense through the oxidation of a thiocyanate ion in the presence of peroxide.[1] The enzyme is composed of a single polypeptide chain with a molecular weight of 78 000, of which about 10% is carbohydrate,[2,3] and a heme group that is extraordinarily tightly bound. Early attempts[4,5] to establish the identity of the prosthetic group used complete pronase digestion of the enzyme. The similarity of the electronic absorption spectrum of the released heme group to that of protoporphyrin IX suggested that a normal protoheme resides at the active site. More recent work based on reductive cleavage of heme with mercaptoethanol in 8 M urea under mild conditions indicated that the prosthetic group is a modified protoporphyrin IX, whose

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Table I. Comparison of the High-Frequency Resonance Raman Bands of the Dioxygen Adducts of Heme Proteins

assgnt	symm	polarizn	freq, cm^{-1}				
			LPO-III[a]	HRP-III[b]	LiP-IIIc[c]	OxyMb[b]	OxyHb[d]
ν(C=C)		p	1623	1637		1619	1620
ν_{10}	B_{1g}	dp	1641	1640	1643	1642	1640
ν_{37}	E_u	p	1613	1601	1604	1603	1606
ν_{19}	A_{2g}	ap	1591	1585	1591	1590	1586
ν_2	A_{1g}	p	1578	1582	1584	1584	1583
ν_{38}	E_u	dp	1556	1543	1555	1549	1552
ν_3	A_{1g}	p	1506	1506	1507	1506	1506
ν_4	A_{1g}	p	1377	1377	1378	1376	1377

[a] This work. [b] Reference 33. [c] Reference 34. [d] Reference 32.

8-methyl group is replaced by an 8-mercaptomethyl group.[6] It was proposed that the unusual heme to apoprotein binding strength is ascribable to the formation of a covalent disulfide bond involving a cysteine residue.

The native lactoperoxidase exists predominantly as a high-spin, five-coordinate heme adduct with a histidine present as the proximal ligand, as evidenced by RR,[7-9] MCD,[10] EPR,[11,12] and NMR[13-17] studies. Although earlier work suggested that the distal site was coordinated by either a carboxylate group of the amino acid residue or a loosely interacting water molecule,[7,10] more recent work has established that the sixth coordination site is vacant and an acid-alkaline transition occurs at pH 12.2.[18]

Lactoperoxidase reacts with hydrogen peroxide or other hydroperoxides to form a primary product, compound I, which decays rapidly in the absence of reducing agents, to generate compound II.[19] Titration of this species with ferrocyanide indicated that compound II contained 2 redox equiv.[20] The observed spectral changes suggested that compound I is a ferrylporphyrin π cation radical, while compound II is a ferrylheme protein-centered radical. The resonance Raman spectrum of lactoperoxidase compound II revealed an unusually low ν(Fe-O) stretching vibration (745 cm^{-1}), which exhibited the expected shift upon ^{18}O substitution. This was accomplished by using $H_2{}^{18}O$ buffer, which was necessary because of the highly efficient ferryl oxygen exchange with the solvent.[21]

The reaction of lactoperoxidase compound II with excess hydrogen peroxide results in the formation of another oxidized derivative, namely, compound III.[19] This species can also be prepared from the oxygenation of the dithionite-reduced ferrous enzyme[22] and the reaction of the ferric protein with enzymatically generated superoxide.[23,24] Unlike oxygen transport proteins such as myoglobin and hemoglobin, which bind oxygen reversibly, the dioxygen molecule in lactoperoxidase compound III was found not to dissociate in a measurable time. In this report, we describe the active-site structural characterization and mechanism of formation of lactoperoxidase compound III prepared by various methods. We also present clear evidence for the vibrational coupling of the Fe-O_2 fragment to a heme mode.

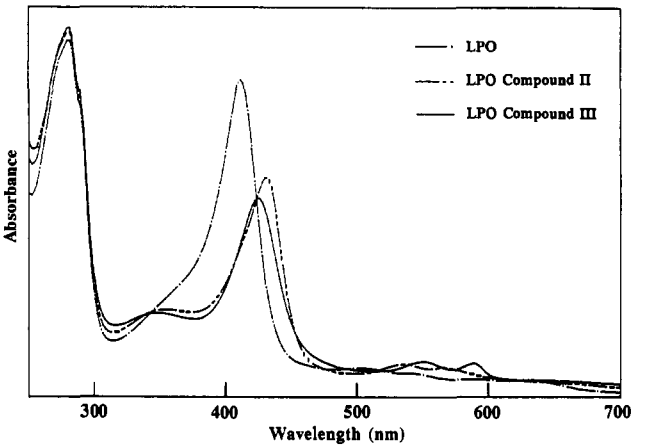

Figure 1. Optical absorption spectra of native lactoperoxidase, lactoperoxidase compound II, and lactoperoxidase compound III in 20 mM potassium phosphate buffer at pH 7.0.

Experimental Procedures

Bovine lactoperoxidase was isolated and purified from fresh cow milk by the methods of Morrison and co-workers,[25,26] with a slight modification. Horseradish peroxidase was purchased from Sigma and purified according to the procedure described by Shannon.[27]

Hydrogen peroxide (30%) and peracetic acid were purchased from Aldrich (Milwaukee, WI) and diluted to desired concentrations. The peracetic acid solution was pretreated with a trace amount of catalase overnight to decompose hydrogen peroxide.[28] The concentration of peroxide was determined spectrophotometrically just before use by using the method based on the LPO-catalyzed oxidation of iodide.[29]

The ^{18}O-labeled hydrogen peroxide was synthesized by the glucose oxidation method,[30] with a modification to avoid the use of toxic sodium cyanide. Specifically, 20 mL of reaction mixture containing 0.1 M glucose and 1 mM EDTA in 10 mM potassium phosphate buffer at pH 7.0 was placed in a 100-mL round-bottom flask, which was then connected to a vacuum line. After the flask was evacuated and filled with pure nitrogen three times to remove oxygen, about 2 mg of glucose oxidase was added. Then, the flask was evacuated and immediately frozen in liquid nitrogen to inactivate the catalase activity and to facilitate the transfer of 60 mL of isotopic oxygen-18 (97%, ICON service, Summit, NJ). After the mixture had been stirred for 3 h at room temperature, the reaction was terminated by adding 0.2 mL of 2 N HCl. The solution was carefully neutralized to pH 7.0 by adding 2 N KOH and was then passed through a 2 × 3 cm column packed with Dowex 1-X

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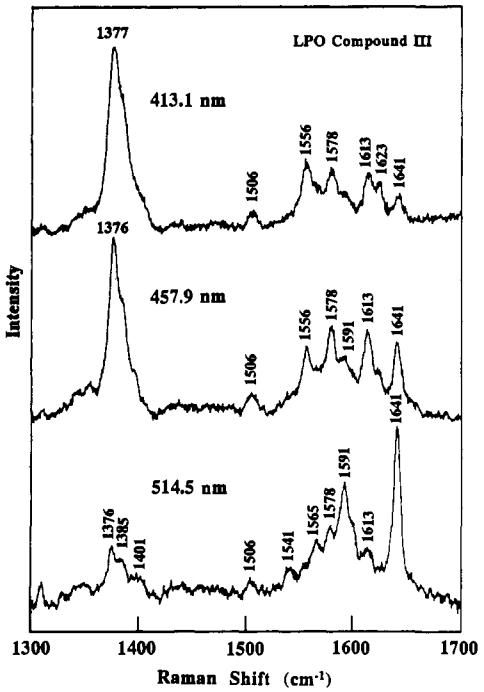

Figure 2. High-frequency resonance Raman spectra of lactoperoxidase compound III prepared by addition of 100-fold H_2O_2.

ion-exchange resin. The concentration of hydrogen peroxide thus obtained was about 80 mM. The isotopic enrichment of the hydrogen peroxide was checked by monitoring the ν(Fe–O) stretching feature of HRP compound II[31] at pH 11.5 and was found to be more than 95% pure.

Resonance Raman spectra were acquired with a Spex 1403 spectrometer equipped with a Hamamatsu R-928 photomultiplier and a Spex DM1B system controller. Excitation lines at 413.1 nm (from a Coherent Innova Model 100-k3 Kr^+ ion laser) and at 457.9 and 514.5 nm (from a Spectra-Physics Model 2025-05 argon ion laser) were used. All the RR spectra presented were acquired with the 413.1-nm excitation line unless otherwise specified. For the study of lactoperoxidase compound III, the NMR tube containing the frozen sample was quickly transferred to a low-temperature double-walled glass cell of in-house design and spectra were recorded in a backscattering geometry. Throughout the spectral acquisition, the NMR tube was kept spinning and the temperature was maintained at −45 °C by flushing the interior of the cell with prechilled dry nitrogen.

Results

The optical spectra of lactoperoxidase and its compound II and compound III derivatives are displayed in Figure 1. The maximum absorption wavelengths are in reasonable agreement with those reported in the literature.[19] It was found that a 100-fold excess of hydrogen peroxide was sufficient to quantitatively convert compound II to compound III under our experimental conditions.

Figure 2 shows the resonance Raman spectra of LPO-III formed by the addition of a 100-fold excess of hydrogen peroxide and stabilized at −45 °C. Several excitation lines were used to selectively enhance both the totally symmetric modes (413.1 and 457.9 nm) and the vibronic modes (514.5 nm). The frequencies are compared in Table I with those of the dioxygen adducts of hemoglobin,[32] myoglobin,[33] horseradish peroxidase,[33] and lignin peroxidase.[34] The assignments of the bands for LPO-III are consistent with the measured depolarization ratios. The Soret-excited spectrum primarily exhibits polarized bands of the A_{1g} modes of the heme group, while the 514.5-nm excitation brings out depolarized and anomalously polarized bands.

The most prominent band in the 413.1- and 457.5-nm-excited spectra (traces A and B of Figure 2) appeared at 1377 cm^{-1}. This band has been assigned to the ν_4 mode, which is described as the C_a–N pyrrole breathing vibration.[35] It has long been known as the oxidation marker band of the heme iron and is particularly sensitive to the electron density of the antibonding π^* orbital of the porphyrin ring. For this reason, it also serves as a π-electron density marker. This mode was previously observed at 1357, 1371, and 1379 cm^{-1} for the ferrous, ferric, and ferryl lactoperoxidases.[8] The frequency shift of the ν_4 band from the native to oxygenated enzyme is comparable to those observed for other heme proteins that possess a histidine axial ligand (see Table I), but it is significantly higher than that detected in the case of cytochrome P450cam,[36] a fact which is consistent with the suggestion that lactoperoxidase contains a histidine axial ligand.

The Raman bands that are sensitive to the spin-state change are the ν_3, ν_{19}, and ν_{10} modes.[35] The ν_{19} band is easily recognized by its high intensity and anomalous polarization with Q-band excitation. This mode was observed at 1562 cm^{-1} for the resting state (at pH 7.0) and 1554 cm^{-1} for the fluoride adduct of lactoperoxidase. Both of these derivatives are formulated as six-coordinate, high-spin-state species.[8] The formation of an authentic six-coordinate, low-spin cyanide adduct shifted the ν_{19} band to 1586 cm^{-1}.[8] Clearly, the observed ν_{19} mode at 1591 cm^{-1} for LPO-III is consistent with its formulation as a six-coordinate, low-spin species. This frequency is also comparable to those detected for many other analogous compounds. For example, the ν_{19} band was observed at 1590, 1586, 1585, and 1591 cm^{-1} for oxyhemoglobin,[32] oxymyoglobin,[33] HRP-III,[33] and LiP-III,[34] respectively.

Another spin-state marker band, ν_{10}, is also readily identifiable in the Q-band-excited RR spectrum, since it is the most prominent band in the whole spectral region. This mode shifted from 1616 (native high-spin enzyme) to 1641 cm^{-1} upon the formation of compound III. The 25-cm^{-1} shift to higher frequency lends further support for the low-spin formulation.

The polarized ν_3 mode, though weak in both the Soret- and Q-band-excited spectrum, is nevertheless easy to identify, owing to its appearance in a less congested spectral region. It is found in the region from ~1470 to ~1510 cm^{-1}, depending on the oxidation and spin state of iron, in various heme proteins and iron porphyrins.[37] Although there is no clear-cut frequency for each set of oxidation and spin states for this mode, a shift to higher frequency is usually observed when a given heme protein undergoes a high- to low-spin-state change.[37] For lactoperoxidase, the ν_3 mode was detected at 1483 cm^{-1} for the native form, 1503 cm^{-1} for the cyanide-bound ferric form, and 1506 cm^{-1} for LPO-III.

In order to further characterize LPO-III, we have obtained the low-frequency RR spectrum where the vibrations of the axial ligands are expected to occur. In Figure 3 are displayed the low-frequency RR spectra of lactoperoxidase compound III and its $^{18}O_2$ analogue. Identical spectral patterns (in both the low- and high-frequency regions) of lactoperoxidase compound III prepared both by the addition of hydrogen peroxide to ferric enzyme (trace A) and by the oxygenation of the ferrous enzyme (trace B) establish the fact that these two preparative methods yield the same product. The strong bands at 679 and 754 cm^{-1} are properly assigned to ν_7 and ν_{16}, respectively.[38] It is noted that a band at 531 cm^{-1} in trace B is absent and a new band at 513 cm^{-1} appears (trace C) when $^{18}O_2$ is used to prepare the oxygenated adduct. The difference spectrum (trace D), obtained by

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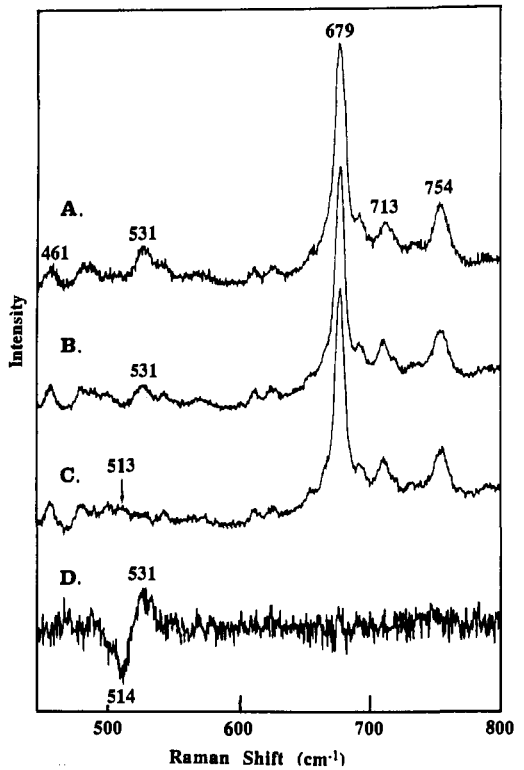

Figure 3. Low-frequency resonance Raman spectra of lactoperoxidase compound III: trace A, addition of 100-fold H_2O_2 to native enzyme; traces B and C, addition of $^{16}O_2$ and $^{18}O_2$, respectively, to dithionite-reduced ferrous enzyme. Trace D: (B - C) × 2.

normalizing traces A and B relative to the ν_7 mode (679 cm^{-1}) and subtracting trace B from trace A, shows clearly that the band at 530 cm^{-1} for the $^{16}O_2$ derivative shifts to 513 cm^{-1} for the $^{18}O_2$ adduct. Thus, we can reasonably assign the 530-cm^{-1} mode to $\nu(Fe-O_2)$, noting that the observed isotopic shift (17 cm^{-1}) is smaller than that based on the isolated two-body vibrator. Such behavior has been previously observed for other metal–axial ligand modes and is indicative of vibrational coupling effects.[39]

We extended the study to LPO-III prepared via the addition of excess H_2O_2 and ^{18}O-labeled H_2O_2, as shown in Figure 4. These spectra were acquired with much longer acquisition times so as to greatly improve the signal-to-noise ratio, which facilitates a detailed inspection of the vibrations of the $Fe-O_2$ linkage. As can be seen, the band at 531 cm^{-1} shifts to 513 cm^{-1} upon the $^{16}O/^{18}O$ isotopic substitution. Careful comparison of traces A and B in Figure 4 reveals another oxygen-isotope-sensitive band at 491 cm^{-1}. The difference spectrum obtained by normalizing traces A and B relative to the band at 461 cm^{-1} shows clearly that this mode shifts to 482 cm^{-1} upon the use of $H_2{}^{18}O_2$ to prepare LPO-III. We tentatively assign this band to the δ(FeOO) mode, noting that the frequency is much higher than the one observed for (dioxygen)(phthalocyaninato)iron(II).[40]

We have assigned the 531-cm^{-1} band to the $\nu(Fe-O_2)$ mode by analogy to Brunner's initial assignment (567 cm^{-1}) for oxyhemoglobin.[41] The expected two low-frequency modes (both the stretching and bending) have thus far eluted detection in the RR spectra of oxygenated heme proteins and iron porphyrins, except for (dioxygen)(phthalocyaninato)iron(II) in a dioxygen matrix at ~15 K as reported by Nakamoto and co-workers.[40] They observed two oxygen-isotope-sensitive bands and were able to reproduce the observed isotopic shift pattern of each mode using a normal-coordinate analysis based on a simple triatomic model. The potential energy distribution supports Brunner's assignment

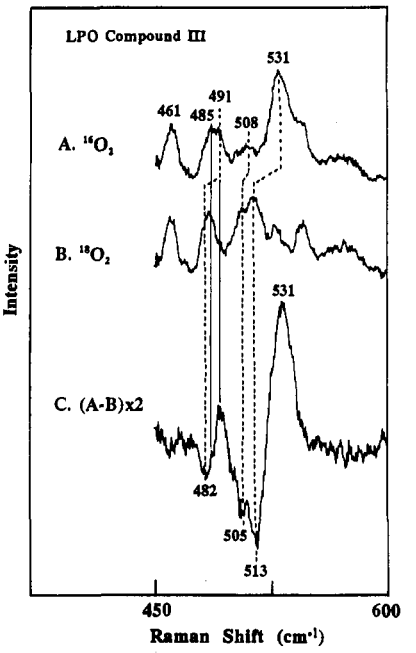

Figure 4. Low-frequency resonance Raman spectra of lactoperoxidase compound III prepared by addition of 100-fold $H_2{}^{16}O_2$ (trace A) and $H_2{}^{18}O_2$ (trace B) to native enzyme. Trace C: (A - B) × 2.

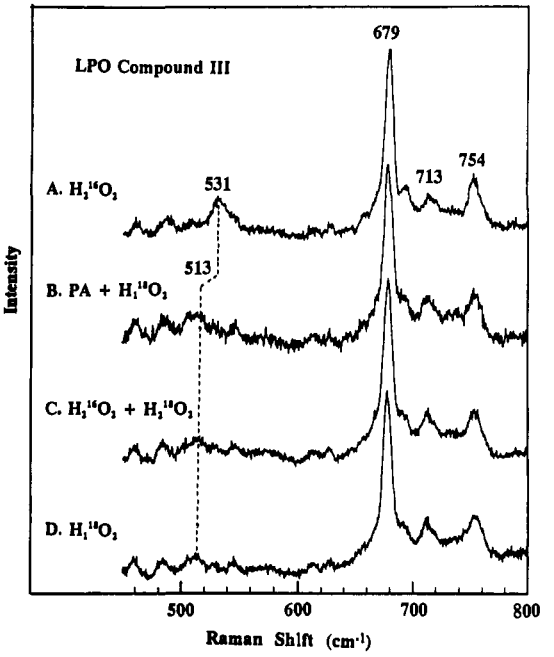

Figure 5. Low-frequency resonance Raman spectra of lactoperoxidase compound III prepared by different combinations of isotopically labeled peroxides as described in the text. PA stands for peroxoacetic acid.

of the ~570-cm^{-1} band to $\nu(Fe-O_2)$.

Figure 5 presents the low-frequency RR spectrum of LPO-III, prepared by using different combinations of isotopically labeled peroxides according to the following reaction schemes:

(a) LPO + $H_2{}^{16}O_2$ (100-fold) → LPO-III

(b) LPO + CH_3COOOH (10-fold) → LPO-II

LPO-II + $H_2{}^{18}O_2$ (100-fold) → LPO-III

(c) LPO + $H_2{}^{16}O_2$ (5-fold) → LPO-II

LPO-II + $H_2{}^{18}O_2$ (100-fold) → LPO-III

(d) LPO + $H_2{}^{18}O_2$ (100-fold) → LPO-III

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The purpose of this set of experiments is to determine whether or not the tightly bound oxygen atom in the ferryl enzyme is retained upon the formation of compound III.[42] Although it is possible to prepare ^{18}O-labeled LPO-II and use it to form compound III with excess $H_2{}^{16}O_2$, the result will not be definitive, because the bound ^{18}O in LPO-II was demonstrated to undergo rapid exchange with the bulk solvent.[21] Thus, this combination is not used in the present study. It is immediately clear that the $\nu(Fe-O_2)$ mode of LPO-III prepared from the addition of excess $H_2{}^{18}O_2$ is attributable to the vibrations of the $Fe-{}^{18}O_2$ linkage.

Peroxidase compound III can be formed by three different routes:[43] (a) the oxygenation of ferrous peroxidase, (b) the reaction of ferric enzyme with superoxide anion, and (c) the reaction of ferryl peroxidase with excess hydrogen peroxide. Although the first two reactions may be considered simply as the binding of the oxygen derivatives to the heme iron, the last one is more complicated because the origin of the bound dioxygen is uncertain. Nevertheless, the present work establishes unambiguously that the ferryl oxygen atom is displaced by the dioxygen in the hydrogen peroxide.

Discussion

A. Heme Core Structure. The marked sensitivity of the high-frequency heme skeletal modes to the oxidation and spin states of central metal ions was first attributed to the changes of heme core size by Yu and co-workers,[44] who, in a study of a number of metallooctaethylporphyrins, noted an inverse correlation between the wavenumbers of the anomalously polarized ν_{19} and the distance from the center of the porphyrin ring to the pyrrole nitrogen (Ct–N). Huang and Pommier[45] later confirmed this observation and found similar dependence for two other bands. More recently, this heme core size correlation has been extended to all of the heme skeletal vibrations above 1450 cm^{-1} and reexamined for a wide range of metalloporphyrins by Spiro and coworkers.[46] By using the established empirical equation $\nu = k(A - d)$, it is possible to calculate the core size (Ct–N distance) from the observed frequency, ν, and the tabulated k and A values.

For native lactoperoxidase, the Ct–N distance of 2.034 Å was obtained from the average of the Ct–N values calculated for the three spin-state marker bands: ν_3 (1483 cm^{-1}), ν_{10} (1616 cm^{-1}), and ν_{19} (1562 cm^{-1}) modes. For the ferrous enzyme, a similar calculation, using the observed ν_3 (1471 cm^{-1}), ν_{10} (1610 cm^{-1}), and ν_{19} (1552 cm^{-1}) bands, yields an average value for the Ct–N distance of 2.055 Å. Upon the formation of lactoperoxidase compound III, the heme core size contracts to 1.980 Å, as computed from the frequencies of the ν_3, ν_{10}, and ν_{19} modes (Table I). This value is comparable to those obtained for oxymyoglobin (1.980 Å), oxyhemoglobin (1.985 Å), HRP-III (1.985 Å), and LiP-III (1.978 Å).[34] Thus, there is little difference in heme core structure, despite the fact that these dioxygen adducts exhibit quite different $\nu(Fe-O_2)$ frequencies.

B. Vibrational Frequency of the $Fe-O_2$ Stretching Mode. Table II compares the frequencies of the $Fe-O_2$ stretching vibration in oxygenated heme proteins and selected ferrous iron porphyrin analogues. It is interesting to note that the observed $\nu(Fe-O_2)$ frequency for lactoperoxidase compound III is considerably lower than those thus far reported for related enzymes and relevant model compounds. This suggests that the $Fe-O_2$ bonding is considerably weakened in this system.

The factors affecting the $Fe-O_2$ stretching frequency in proteins include the trans-ligand effect, the heme pocket environment, and the geometry of the $Fe-O_2$ linkage. These have been discussed extensively by Yu et al.[47] and by Babcock et al.[48] for the iron–oxygen vibrations. Recently, we have detected a similarly low $\nu(Fe-O_2)$ frequency in the resonance Raman spectrum of oxygenated cytochrome P450cam,[36] which possesses a mercaptide axial ligand that can donate "extra" electrons so as to weaken the trans $Fe-O_2$ bonding. However, it has been shown that lactoperoxidase contains a histidine residue as the endogenous ligand and is thus similar to myoglobin and horseradish peroxidase. The recently reported resonance Raman spectrum of the ferrous derivative revealed the presence of an unusually strong iron–histidine linkage, as reflected by the occurrence of the ν(Fe–His) mode at 255 cm^{-1}.[8] This mode has been detected at 220 cm^{-1} for ferrous myoglobin[49] and 244 cm^{-1} for ferrous HRP.[50] However, the strong iron–histidine bond is expected to strengthen the $Fe-O_2$ bonding interaction and increase the $\nu(Fe-O_2)$ frequency,[48] inasmuch as the proximal imidazole of anionic character will increase the π-donation to the $O_2(\pi^*)$ orbital via the $Fe(d_\pi)$. It is suggested that, when O_2 binds to the heme pocket of a peroxidase, the electron density on the antibonding π^* orbital of the O=O bond is pulled out by the action of a positively charged arginine residue on the distal side.[48] This interaction will result in the strengthening of O=O bonding and concomitant weakening of the $Fe-O_2$ linkage. In addition, the reduced electron density on the $O_2(\pi^*)$ orbital is expected to open the angle of the Fe–O–O fragment,[51] which, by kinematic effect, will further lower the $\nu(Fe-O_2)$ frequency.[52] It is plausible that the distal effect on the $Fe-O_2$ unit in lactoperoxidase compound III overrides the trans-ligand effect, thus accounting for an unusually low $\nu(Fe-O_2)$ frequency (531 cm^{-1}).

C. Vibrational Coupling of the $Fe-O_2$ Fragment with Heme Modes. Careful examination of the $\nu(Fe-O_2)$ region of the RR spectrum of LPO-III provides direct evidence for vibrational coupling of $\nu(Fe-O_2)$ with a low-frequency heme mode, which, in turn, results in a nonideal isotopic shift. Thus, it is noted that the 531-cm^{-1} band in trace A of Figure 4 shifts to 513 cm^{-1}. However, its intensity is diminished relative to the 531-cm^{-1} feature and a clearly resolved shoulder at 505 cm^{-1} emerges for the $^{18}O_2$ derivative (trace B). This complicated spectral pattern can be reasonably explained by invoking the following vibrational coupling scheme.

Table II. Vibrational Frequencies (cm^{-1}) of the $Fe-O_2$ Linkage in Oxygenated Heme Proteins and Selected Analogues[a]

	$\nu(Fe-O_2)$, cm^{-1}	ref
LPO-III	531	this work
cytochrome P450cam	541	36
HRP-III	562	33
LiP-III	563	34
Hb	567	41
Mb	570	59
reduced CcO	568–571	60
mixed-valence CcO	568–572	61
(NMI)$Fe^{II}-O_2$(PP)	573	48
(NMI)$Fe^{II}-O_2$(PA)	576	48
(PIP)$Fe^{II}-O_2$(TPP)	575	62

[a] Abbreviations: Hb, hemoglobin; Mb, myoglobin; CcO, cytochrome *c* oxidase; HRP-III, horseradish peroxidase compound III; LPO-III, lactoperoxidase compound III; LiP-III, lignin peroxidase compound III; NMI, *N*-methylimidazole; PIP, piperidine; PP, protoporphyrin IX dimethyl ester; PA, heme *a*; TPP, tetraphenylporphyrin.

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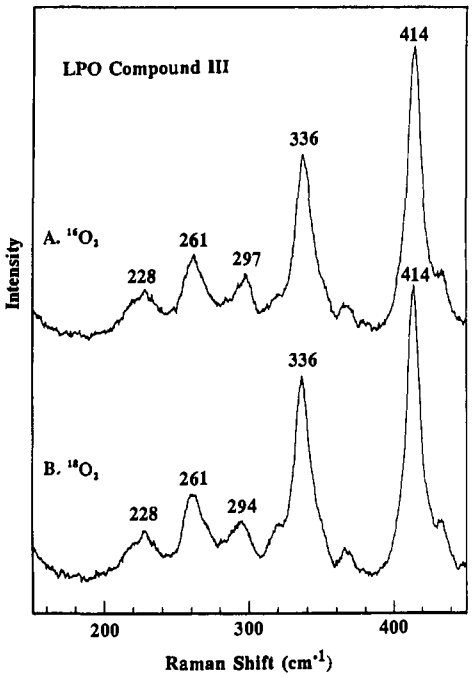

Figure 6. Resonance Raman spectra of lactoperoxidase compound III prepared by addition of 100-fold $H_2{}^{16}O_2$ (trace A) and $H_2{}^{18}O_2$ (trace B) to the native enzyme.

For the Fe–O_2 fragment, the unperturbed isotopic shift is expected to lie between 19 and 23 cm^{-1}, a value calculated on the basis of a harmonic two-body vibrator by assuming the dynamic mass of the bound dioxygen to be 16 and 32 amu, respectively. The actual value depends on the angle of the Fe–O–O unit and is likely to be 21 cm^{-1}, if the angle is assumed to be about 145° as revealed by the X-ray crystallographic determinations of the dioxygen adducts of iron porphyrins[53] and heme proteins.[54] Thus, the 531-cm^{-1} band is expected to shift to 510 cm^{-1} upon the $^{16}O_2/^{18}O_2$ substitution. These considerations imply that the inherent frequency of the ν(Fe–O_2) mode is 510 cm^{-1}. We note, however, that a low-frequency heme core mode occurs at 508 cm^{-1} (Figure 4, trace A) which is closely energy matched with this inherent frequency. This condition facilitates vibrational coupling of the two modes, giving rise to two frequencies shifted by ±3 cm^{-1} (i.e., 510 → 513 and 508 → 505 cm^{-1}). In agreement with theory,[39] the total intensity of the 505/513-cm^{-1} doublet is within experimental error of the integrated intensity of the 531-cm^{-1} feature.

It is worthwhile pointing out that this vibrational coupling scheme can also be applied to account for the previously unexplained anomalous behavior observed in the low-frequency RR spectra of the $Co^{II}(T_{piv}PP)(1,2\text{-}Me_2Im)O_2$ adduct reported by Yu and co-workers.[55] In that case, the ν(Co–O_2) was identified at 527 cm^{-1} and was suggested to shift to 498 cm^{-1} upon the oxygen isotopic exchange. Obviously, this shift (29 cm^{-1}) is much larger than that expected (19–23 cm^{-1}); i.e., the expected inherent frequency of ν(Co–$^{18}O_2$) is ~506 cm^{-1}. Careful examination of the spectrum reveals a feature at about 504 cm^{-1}. (Since the band was not labeled, the number was estimated relative to the bands at 498 and 527 cm^{-1}. This may introduce some error in the location of the exact frequency.) The energy of this band matches closely that of the unperturbed ν(Co–$^{18}O_2$) mode at 506 cm^{-1}. It is plausible that they may interact to give rise to the observed spectral doublet at 498 and 515 cm^{-1}.

It is necessary to point out that we observed one additional feature which is sensitive to oxygen-isotope substitution. Specifically, we note that a band at 297 cm^{-1} shifts 3 cm^{-1} to 294 cm^{-1} upon the $^{16}O_2/^{18}O_2$ substitution, as is shown in Figure 6. Previously, a similar mode was assigned to the iron–histidine stretching vibration of the cyanide and carbon monoxide adducts of heme proteins[56] on the basis of the fact that it shows isotopic sensitivity to both the iron and axial ligands. However, in our recent RR study of oxyhemoglobin,[57] it was found that a mode at 298 cm^{-1} exhibits a similar oxygen isotopic shift. Moreover, it also downshifts 6 cm^{-1} upon meso-deuteration of protoporphyrin IX. The combined results suggest that the mode in question arises from a heme out-of-plane vibration, involving mainly the motion of methine carbon atoms,[58] and argues against its assignment to the iron–histidine mode. We propose that the observed isotopic sensitivity can be attributed to coupling of this out-of-plane mode with the δ(FeOO) vibration.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (DK35153).

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