Heme–Heme Oxygenase Complex: Structure and Properties of the Catalytic Site from Resonance Raman Scattering†

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ABSTRACT: The resonance Raman spectra of ferric and ferrous forms of the heme–heme oxygenase (HO) complex (isoform 1) clarify several structural features of the catalytic active site. Isotopic substitution studies of the central iron atom of the heme demonstrate that the line at 218 cm^{-1} in the ferrous ligand-free form of the complex originates from the iron–histidine stretching mode. The presence of a Raman line at this frequency confirms that the fifth ligand coordinating to the heme is a neutral imidazole from a histidine residue. The modes associated with CO in the carboxy derivative of the ferrous enzyme complex have typical frequencies of histidine-bound heme proteins such as myoglobin. In the ferric form of the complex, at alkaline pH, hydroxide is identified as the bound exogenous ligand, and at neutral pH we infer that water is bound. Thus, the coordination of the heme–HO complex is the same as that in myoglobin. However, in a comparison of the low-frequency vibrational modes in the resonance Raman spectrum of the heme–HO complex to those of myoglobin, the spectra are found to be very different, indicating that the interactions between the heme and its amino acid pocket in these two proteins are quite different. The neutral imidazole may play several important roles in the physiological function of the heme–HO complex.

Heme oxygenase (HO) is the first and rate-limiting enzyme of the microsomal heme degradation pathway. The catabolism of heme *b* (iron protoporphyrin IX) by HO and oxygen cleaves the heme at the α-meso position and generates carbon monoxide (CO) and biliverdin IX-α (Tenhunen et al., 1968, 1969; O'Carra,1975). Three oxygen atoms are used for the ring opening (one for carbon monoxide, two for biliverdin IX-α) which are derived from different oxygen molecules (Tenhunen et al., 1972; Brown & King, 1976) and are incorporated into substrate by successive cycles of monooxygenase reactions (Yoshida & Kikuchi, 1978b; Kikuchi & Yoshida, 1983). HO has two known isoforms, referred to as HO-1 and HO-2 (Maines et al., 1986), which are encoded by different genes (Muller et al., 1987; Cruse & Maines, 1988). HO-1, which has a molecular mass of about 33 kDa and is highly expressed in spleen and liver, is inducible and responsible for the heme degradation mainly in the catabolism of hemoglobin. HO-2, with a molecular mass of 36 kDa, is a constitutive enzyme and is found in the brain as well as in several other organs (Maines, 1988). It was reported recently that the CO generated by HO-2 may be a neurotransmitter and can activate guanylyl cyclase, just as nitric oxide can (Brune & Ullrich, 1987; Verma et al., 1993). The amino acid sequence homology between isoforms 1 and 2 is about 40%, but the predicted structures of both isoforms coincide (Rotenberg & Maines, 1991). Thus, it is generally assumed that the two isoforms of HO have homologous active site structures and reaction mechanisms.

HO contains no prosthetic groups, but heme forms a 1:1 complex with HO and serves both as the enzyme substrate and as a cofactor of the enzyme (Yoshida et al., 1974; Yoshida & Kikuchi, 1978a). The heme–HO complex has properties analogous to those of hemoproteins exhibiting ferric and ferrous redox states, binding exogenous inhibitory ligands such as CO or CN^-, and having characteristic hemoprotein absorption spectra (Yoshida & Kikuchi, 1978a, 1979). Formation of a metastable ferrous oxygen binding form was also demonstrated (Yoshida et al., 1980). Hence, although the substrate for HO is the bound heme, the enzyme–substrate complex can be considered a member of the heme-containing class of monooxygenases. The largest class of monooxygenases are the cytochrome P-450's (Ortiz de Montellano, 1986; Waterman & Johnson, 1991). Both of the enzymes accept molecular oxygen as a ligand in the ferrous state, which is activated for oxygen transfer by an electron donated by NADPH–P450 reductase. However, it has been proposed that the nature of this activated complex and the mechanism of the oxygenation reactions may be quite different in the two distinct types of enzyme (Wilks & Ortiz de Montellano, 1993; Takahashi et al., 1994).

Until recently, spectroscopic investigation of the heme–HO complex has been limited to optical absorption studies due to the small amounts of enzyme available. However, the recent application of biotechnology has led to the preparation of large amounts of the HO-1 isoform by bacterial overexpression (Ishikawa et al., 1991, 1992). Furthermore, trypsin digestion of the enzyme cleaves the hydrophobic sequence

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near the C-terminal end (Yoshida et al., 1991), which is involved in membrane binding (Yoshida & Sato, 1989), yielding a catalytically active 28-kDa water-soluble form and thereby enabling spectroscopic studies to be carried out with relative ease. In a preliminary study of this form of HO-1 by optical absorption, EPR, and resonance Raman scattering, we found that the ferric enzyme shows a pH-induced spin-state transition with a pK_a of 7.6, and the ferrous enzyme is five-coordinate with a line at 218 cm^{-1} which we proposed originates from an iron–histidine stretching mode (Takahashi et al., 1994). Similar conclusions were drawn in an independent study of the EPR and resonance Raman spectra (Sun et al., 1994).

In this paper we report a comprehensive study of the resonance Raman spectra of the HO-1 soluble derivative. By isotopic substitution of the iron atom we are able to confirm that the line at 218 cm^{-1} in the ferrous enzyme is the iron–histidine stretching mode; that in the CO form of the enzyme the Fe–CO stretching mode, the Fe–C–O bending mode, and the C–O stretching mode are located at 503, 576, and 1958 cm^{-1}, respectively; and that in the alkaline form of the ferric enzyme the sixth ligand is a hydroxide ion. The comparison of these lines with corresponding lines from other heme proteins confirms that the heme is coordinated by a neutral imidazole. This is in striking contrast to most other peroxidases and oxygenases, which have anionic axial ligands and a polar distal pocket to facilitate oxygen–oxygen bond cleavage. Thus, although the apparent function and overall reaction scheme of HO are similar to those of cytochrome P-450's, the oxygen-activation mechanisms of the enzymes appear to be quite different. In part, the mechanism of oxygen activation in P-450's is a consequence of the electronic properties of the thiolate ligand which coordinates the heme. Although the coordination of the heme in the heme–heme oxygenase complex is the same as that in myoglobin, the low-frequency modes in the resonance Raman spectrum demonstrate that the heme pockets are quite different. HO falls into a small class of known mono- and dioxygenase heme enzymes with a neutral fifth ligand.

MATERIALS AND METHODS

The water-soluble form of rat heme oxygenase 1, which lacks the hydrophobic C-terminal 26 amino acid sequence that anchors the enzyme to the microsomal membrane, was used in this study (Takahashi et al., 1994). This derivative retains the same heme oxygenase activity as the original enzyme in converting hemin to biliverdin (Ishikawa et al., 1992). The detailed preparative procedures are described elsewhere (Ishikawa et al., 1992; Takahashi et al., 1994). Myoglobin from horse skeletal muscle was purchased (Sigma, St. Louis, MO) and used without further purification. Sample solutions for the Raman measurements contained 50 μM HO or 100 μM myoglobin in 100 mM sodium phosphate (pH 6.5), Tris-HCl (pH 9.0), or CAPS–NaOH (pH 10.5) buffer and were sealed in a rotating cell (ca. 1000 rpm). The ferrous heme-bound enzyme complex was obtained by adding a fresh solution of sodium dithionite to the ferric enzyme under a nitrogen atmosphere. The final concentration of the reductant was about 1 mM. The CO-bound form of the complex was prepared by flushing CO over the ferrous sample. Formation of each redox and ligand-bound state and sample integrity after laser exposure for the Raman measurements were confirmed by measuring the optical absorption spectrum of the sample before and after the Raman measurements. The $H_2{}^{18}O$-substituted sample was prepared by dissolving the concentrated enzyme complex in buffer made with the isotopic water (ICON, Mt. Marion, NY). The isotopic substitution ratio was about 90%. The ^{54}Fe- and ^{57}Fe-incorporated samples were prepared by adding isotopically labeled hemin (Porphyrin Products, Logan, UT) into the enzyme.

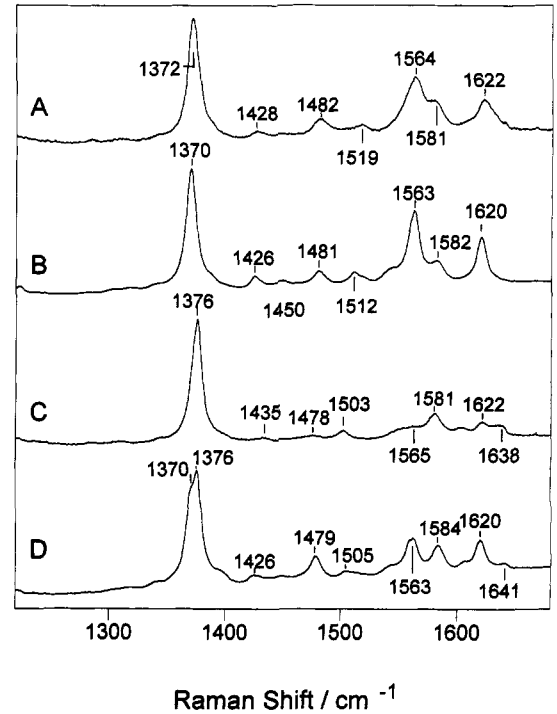

FIGURE 1: Resonance Raman spectra of the high-frequency region of the ferric heme–HO complex and ferric myoglobin at neutral and high pH. (A) Ferric heme–HO at pH 6.5. (B) Ferric Mb at pH 6.5. (C) Ferric heme–HO at pH 9.0. (D) Ferric Mb at pH 10.5. The laser excitation wavelength was 413.1 nm.

Raman scattered light was dispersed by a single polychromator (SPEX, Metuchen, NJ, Model 1269) equipped with a 1200 grooves/mm grating and detected by a cooled CCD camera (Photometrics, Tucson, AZ, Model CCD9000). The spectral slit width was about 5 cm^{-1}. The Rayleigh scattering was removed by holographic filters (Kaiser, Ann Arbor, MI). The excitation wavelengths were 413 nm, obtained from a krypton ion laser (Spectra Physics, Mountain View, CA), and 442 nm, obtained from a helium–cadmium vapor laser (Liconix, Santa Clara, CA). The power at the sample was about 30 mW unless otherwise stated. Typically, several 1-min spectra were obtained and averaged after eliminating spikes caused by cosmic rays. Spectral shifts were calibrated against the Raman spectra of neat carbon tetrachloride, acetone, and indene and an aqueous solution of ferrocyanide.

RESULTS

Ferric Heme–Enzyme Complex. In Figure 1 the high-frequency region of the resonance Raman spectra of the ferric heme–HO complex at low and high pH are compared to the corresponding spectra of ferric myoglobin. The same comparison in the low-frequency region of the spectrum is reported in Figure 2. It is well established that the lines in the high-frequency region of the Raman spectrum of ferric hemoproteins are sensitive to the spin state of the central iron atom. Specifically, ν_2 and ν_3 have distinct frequencies for five-coordinate high spin (1570–1575 and 1490–1500 cm^{-1}, respectively), six-coordinate high spin (1560–1565 and 1475–1485 cm^{-1}, respectively), and six-coordinate low spin (1580–1590 and 1500–1510 cm^{-1}, respectively) (Spiro et al., 1979).

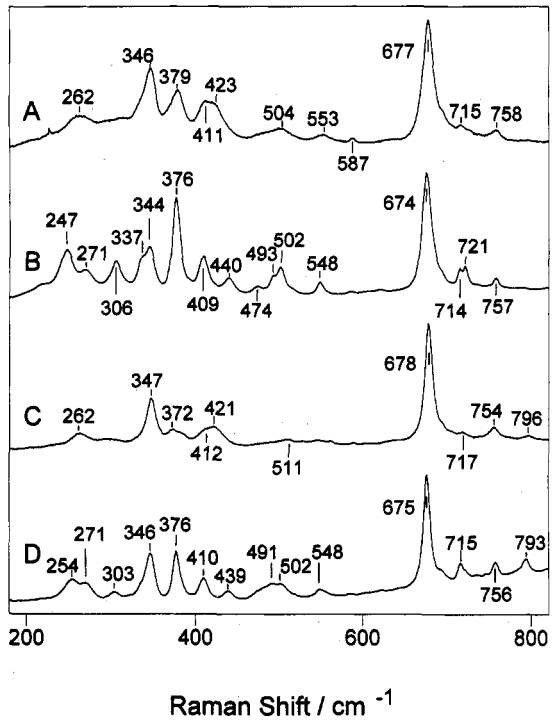

FIGURE 2: Resonance Raman spectra of the low-frequency region of the ferric heme–HO complex and ferric myoglobin at neutral and high pH. (A) Ferric heme–HO at pH 6.5. (B) Ferric Mb at pH 6.5. (C) Ferric heme–HO at pH 9.0. (D) Ferric Mb at pH 10.5. The laser excitation wavelength was 413.1 nm.

As we have reported previously, the pH-induced change in the spectra of the heme–HO complex clearly demonstrates the transition from a primarily six-coordinate high-spin form to a form that is primarily six-coordinate low spin (Takahashi et al., 1994). At low pH the spectrum of the heme–HO complex agrees well with that of ferric myoglobin, both being six-coordinate high spin. However, at pH 10.5, myoglobin is mixed spin as evidenced by the appearance of two lines for ν_3 (1479 and 1505 cm^{-1}) and ν_4 (1370 and 1376 cm^{-1}) in its spectrum in Figure 1, whereas the heme–HO complex has undergone a near complete spin conversion by pH 9 to a low-spin form.

The major change in the low-frequency spectrum of the heme–HO complex in going from low to high pH is the loss of intensity in the line at 379 cm^{-1}. In the spectrum of myoglobin, this line at 376 cm^{-1} also decreases substantially at alkaline pH. There are other, smaller relative intensity and line-width changes in the low-frequency spectrum. For example, the line widths of the ring stretching modes (ν_7 at 677 cm^{-1} and ν_8 at 346 cm^{-1}) narrow from 15 and 15 cm^{-1} to 10 and 12 cm^{-1} (full width at half-height), respectively, with an increase in the solution pH. We attribute these changes primarily to the change in spin equilibrium. The low-frequency spectra of the heme–HO complex and myoglobin are very different at both pHs. Although the frequencies for the ring vibrational modes, such as ν_7 and ν_8, are located at similar positions in these two proteins, those for the vibrations which have out-of-plane or peripheral substituent character show large differences. It is also noted that the line widths of the lines which show frequency deviations between the two proteins also become substantially broader for the heme–HO complex. The possible assignments of the low-frequency modes are summarized in Table 1.

In the hydroxide form of many heme proteins, Fe–OH stretching modes have been detected (Asher et al., 1977; Asher & Schuster, 1979; Sitter et al., 1988). To determine whether

Table 1: Comparison of the Low-Frequency Modes of Ligand-Free Ferrous, CO-Bound Ferrous, and Ferric Derivatives of the Heme–Heme Oxygenase Complex and Myoglobin[a]

assignment	HO Fe^{2+}	Mb Fe^{2+}	HO $Fe^{2+}CO$	Mb $Fe^{2+}CO$	HO Fe^{3+}	Mb Fe^{3+}
Fe–His stretch	218	220				
pyrrole tilt	255	243		253		246
					262	269
vinyl bend; methine C OoP[b]	301	306		316		304
						335
ν_8 (IP substituent bend)	340	344	339	345	346	344
ν_8 or ν_{35}	350		353			
peripheral OoP[b]	360	372		377	379	376
vinyl bend	411			409	411	409
pyrrole fold			427	434	423	439
						473
	497	500			504	493/501
Fe–CO stretch			503	507		
	548				553	548
Fe–C–O bend			576	577		
ν_{48}	588				587	584
ν_7	673	674	676	676	677	674
	720		719		715	714
						721
ν_{16} (pyrrole deformation)	757	757	754	754	758	758

[a] The mode assignments are taken from Abe et al. (1978) and Spiro and Li (1988). [b] OoP refers to out-of-plane.

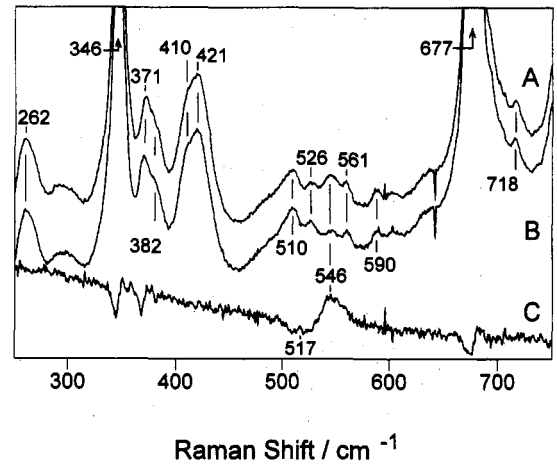

FIGURE 3: Resonance Raman spectra of the ferric heme–HO complex at pH 9.0. Trace A was obtained for the sample dissolved in $H_2{}^{16}O$ buffer, and trace B was obtained in $H_2{}^{18}O$ buffer. Trace C is the difference spectrum of trace A minus trace B. The laser excitation wavelength was 413.1 nm.

hydroxide is the sixth ligand in the alkaline form of the heme–HO complex, we have measured the spectrum of the complex in the presence of $H_2{}^{18}O$ and compared it to that in the presence of $H_2{}^{16}O$ (Figure 3). The derivative pattern of the difference spectra allows the identification of a line at 546 cm^{-1} for $H_2{}^{16}O$ as an Fe–^{16}OH stretching mode. We carefully compared the spectrum in the Fe–OH region to those of the alkaline form of myoglobin (S. Takahashi and D. L. Rousseau, manuscript in preparation) and other heme proteins as summarized in Table 2. From this comparison we are able to identify the mode at 546 cm^{-1} in the heme–HO complex as originating from a low-spin form of the complex. The relatively high frequency for the Fe–OH stretching mode in the low-spin form of myoglobin as compared to that in horseradish peroxidase is attributed to coordination by a neutral imidazole trans to the hydroxide bond (S. Takahashi and D. L. Rousseau, manuscript in preparation).

Exogenous Ligand-Free Ferrous Heme–Enzyme Complex. Figure 4 shows high-frequency resonance Raman spectra of the ferrous heme–HO complex. The spectrum, in this region

Table 2: Comparison of the Fe–OH Stretching Modes of Several Different Heme Proteins

protein	spin state	Fe–OH frequency (cm^{-1})	reference
Hb	HS	495	Asher et al. (1977)
	LS	not detected	
Mb	HS	490	Asher and Schuster (1979)
	LS	551	Takahashi and Rousseau, unpublished
CcO	?	477	Han et al. (1989, 1990)
		450	
HRP	LS	503 (isozyme C)	Sitter et al. (1988)
		516 (isozyme A-1)	
heme–HO complex	LS	546	this work

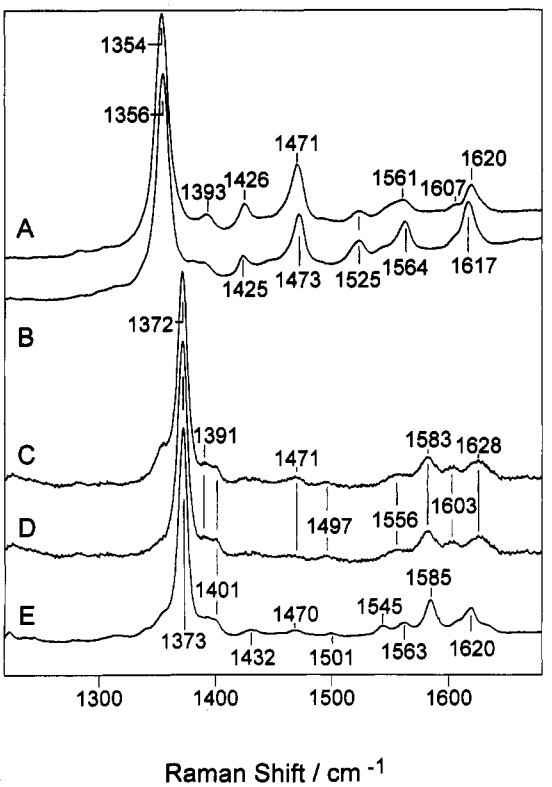

FIGURE 4: Resonance Raman spectra of the high-frequency region of the ligand-free ferrous and the CO-bound ferrous forms of the heme–HO complex and myoglobin. (A) Ligand-free ferrous heme–HO complex. (B) Ligand-free ferrous Mb. (C) CO-bound ferrous derivative of the heme–HO complex. (D) Trace C after subtraction of a small contribution from the ligand-free enzyme due to photodissociation. (E) CO-bound ferrous derivative of Mb. All samples were dissolved in pH 6.5 buffer. The power of the excitation laser (413.1 nm) for the CO-bound derivatives was about 5 mW for the heme–HO complex and 1 mW for Mb.

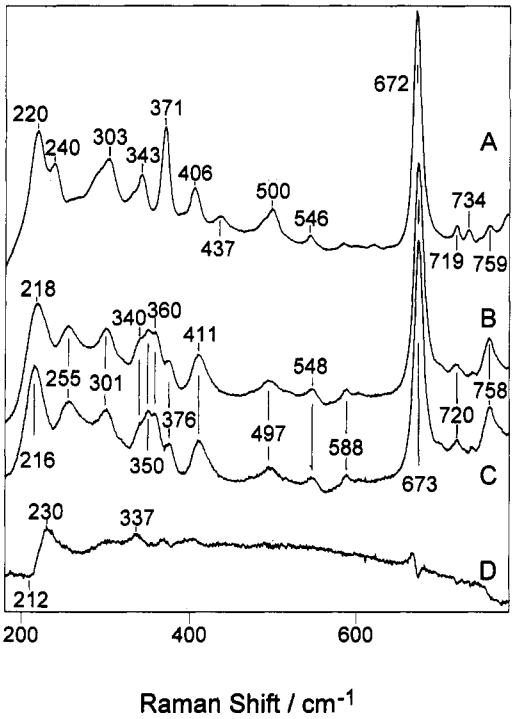

FIGURE 5: Resonance Raman spectra of the low-frequency region of the ligand-free ferrous heme–HO complex and ligand-free ferrous Mb. (A) Ligand-free ferrous Mb at pH 6.5. (B) Ligand-free ferrous heme–HO complex at pH 6.5. (C) Ligand-free ferrous heme–HO complex at pH 9.0. (D) Difference spectrum of trace B minus trace C. The excitation laser wavelength was 441.6 nm.

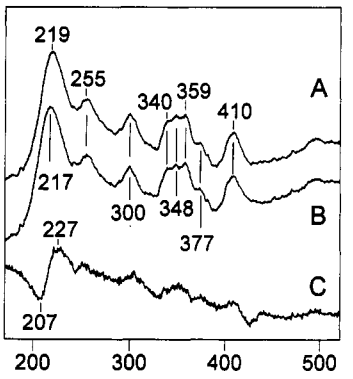

FIGURE 6: Resonance Raman spectra of the low-frequency region of the ligand-free ferrous heme–HO complexes with ^{54}Fe-incorporated hemin (trace A) and ^{57}Fe-incorporated hemin (trace B). Trace C shows the difference spectrum between traces A and B. The excitation wavelength was 441.6 nm.

of the spin- and coordination-sensitive lines, is very similar to that of ferrous five-coordinate myoglobin. The frequencies of the ν_2, ν_3, and ν_4 modes at 1561, 1471, and 1354 cm^{-1}, respectively, demonstrate that the ferrous form of the heme in the HO complex is also five-coordinate high spin. No pH change was observed in this region. The low-frequency resonance Raman spectrum of the ferrous heme–HO complex is compared to that of myoglobin in Figure 5. Just as was found in the ferric forms of the complex, the spectrum is very different from that of the corresponding form of myoglobin. Major differences occur in the line at 240 cm^{-1} in deoxyMb, which appears at 255 cm^{-1} in the heme–HO complex; in the 340–380-cm^{-1} region, which is completely different in these two proteins; and in the line at 437 cm^{-1}, which is absent in the heme–HO complex.

The iron–histidine stretching mode of five-coordinate heme proteins is found in the 200–250-cm^{-1} region (Kitagawa, 1988). Therefore, in our prior study we proposed that the mode at 218 cm^{-1} in the ferrous ligand-free heme–HO complex was an Fe–His stretching mode (Takahashi et al., 1994). To confirm this speculation, we compared the Raman spectrum of HO complexed with [^{54}Fe]hemin to that of [^{57}Fe]hemin. The line at 219 cm^{-1} shifts by about 2 cm^{-1} as is evident in the spectra of each isotope in Figure 6 and as can be seen clearly in the difference spectrum. Assuming that the Fe–His stretching mode has a Gaussian line shape, as is the case for hemoglobin (D. L. Rousseau, unpublished results), difference spectra analysis (Rousseau, 1981) gives a frequency shift of 1.8 cm^{-1}. A shift of about 2 cm^{-1} is the calculated shift if the approximation is made that the iron and the

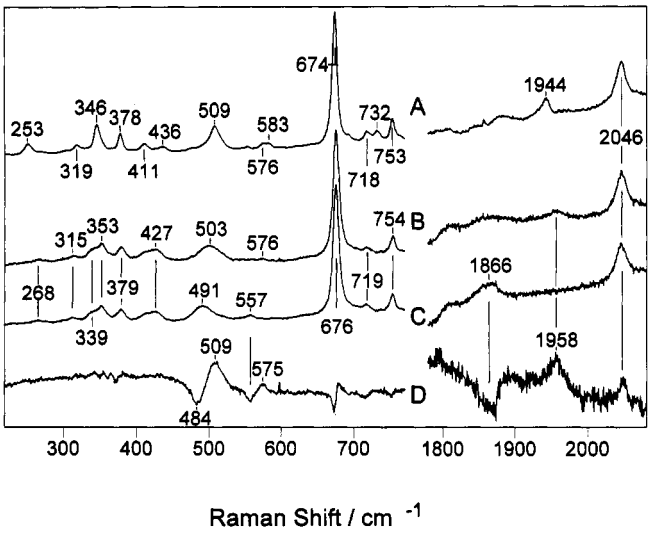

FIGURE 7: Resonance Raman spectra of the Fe–CO stretching mode and the C–O stretching mode regions of the CO-bound ferrous heme–HO complex (pH 9.0) and myoglobin (pH 6.5). Traces A–D correspond to Mb–$^{12}C^{16}O$, heme–HO–$^{12}C^{16}O$, heme–HO–$^{13}C^{18}O$, and the difference spectrum between traces B and C, respectively. The excitation wavelength was 413.1 nm with a power of about 5 mW for the heme–HO complexes and about 1 mW for Mb.

imidazole ring of the histidine may be treated as a simple diatomic species. The Fe–His stretching mode also shifts as the pH is increased. In Figure 5 the low-frequency spectra of the complex at pH 6.5 and 9.0 are compared. The Fe–His stretching mode shifts by about 2 cm^{-1} to lower frequency as the pH is increased. Small intensity changes in the 340–380-cm^{-1} region are also associated with this pH change. A pH sensitivity of the Fe–His stretching mode is not observed in myoglobin, but larger shifts (3–14 cm^{-1}) in the same direction are usually seen in the spectra of peroxidases (Teraoka & Kitagawa, 1981; Teraoka et al., 1983; Hashimoto et al., 1986).

CO-Bound Ferrous Heme–Enzyme Complex. The high-frequency resonance Raman spectrum of the CO-bound ferrous complex, shown in Figure 4, has ν_2, ν_3, and ν_4 lines at 1583, 1497, and 1372 cm^{-1}, respectively, characteristic of a CO-bound low-spin heme. However, the comparison of this spectrum to that of CO-bound myoglobin reveals some clear differences in the 1600–1640 cm^{-1} region. In MbCO distinct lines from the vinyl peripheral substituent at about 1620 cm^{-1} and from ν_{10} at about 1638 cm^{-1} can be identified. In contrast, in the heme–HO complex a single broad line centered at 1628 cm^{-1} is found. Three isotope-sensitive lines, shown in Figure 7, were observed for the CO-bound complex in the comparison of the $^{13}C^{18}O$ derivative to the $^{12}C^{16}O$ derivatives. The line at 503 cm^{-1} can be assigned as the Fe–CO stretching mode; that at 576 cm^{-1}, as the Fe–C–O bending mode; and that at 1958 cm^{-1}, as the C–O stretching mode. The position of the Fe–CO stretching mode is slightly lower than those observed for native hemoglobins and myoglobins (509 cm^{-1}), the Fe–C–O bending mode is at the same frequency, and the C–O stretching mode is somewhat higher than that in MbCO (1944 cm^{-1}). The intensity of the bending mode is smaller than that in MbCO. The frequency of the Fe–CO stretching mode and the intensity of the Fe–C–O bending mode have been shown to correlate with the orientation of the bound CO in other heme proteins and model compounds (Yu et al., 1983). Compared to those of MbCO, the lower frequency of the Fe–CO stretching mode and the lower intensity of the Fe–C–O bending mode are indicative of a less bent or tilted orientation of the CO in the heme–HO complex. It should be noted that whereas the line widths for the porphyrin ring modes (ν_4 or ν_7, for example) become narrower upon CO coordination, the line widths for the Fe–CO and C–O stretching modes are quite broad, being about 30 cm^{-1} for the Fe–CO stretching mode and 21 cm^{-1} (full width at half-height) for the C–O stretching mode. These broad lines suggest that the environment for the bound CO is heterogeneous.

Unlike the results for the other forms of the enzyme, no pH dependence was detected in the high- and low-frequency regions of the CO-bound form (data not shown). It has been established that the rate of geminate rebinding of the ligand after photolysis is sensitive to the properties of the distal pocket (Ikeda-Saito et al., 1993). Experimentally, the effect of changes in the geminate rebinding rate influences the apparent ease of photodissociation of solution samples in a rotating cell when illuminated by the laser beam. Since the sample residence time in the excitation laser beam is about 10 μs with our experimental conditions, we can ignore the bimolecular CO binding precess, which usually occurs on the millisecond time scale under 1 atm of CO pressure. We have compared the amounts of photodissociation at various laser powers as a function of pH. In the comparison of low to high pH we find no change in the degree of photodissociation. This result has been confirmed by kinetic absorption measurements (E. E. Clifford and M. Ikeda-Saito, unpublished results). Qualitatively, the degree of photodissociation in the CO-bound form of the heme–HO complex is the same as that observed in myoglobin under the same conditions.

DISCUSSION

Axial Ligands and Coordination States. The resonance Raman data allow unambiguous determination of axial ligands in several states of the heme–HO complex. The spectrum of the ferrous exogenous ligand-free form of the complex shows that this form is five-coordinate high spin just as in hemoglobin and myoglobin. The assignment of the iron–histidine stretching mode of the complex by isotopic substitution of the iron atom proves that the heme is coordinated to the protein by a histidine residue. Its frequency is consistent with neutral imidazole coordination rather than imidazolate coordination as occurs in peroxidases (Kitagawa, 1988). Neutral imidazole coordination is also found in the CO-bound form of the complex. There is a well-established correlation between the frequencies of the Fe–CO stretching mode and the C–O stretching mode spanning many different heme proteins and model complexes as shown in Figure 8 (Uno et al., 1987; Yu & Kerr, 1988). The values we measured for the heme–HO complex place it on the lower half of the correlation line for imidazole coordination, where most myoglobins and hemoglobins are located. An additional indication of the coordination is given by the frequency of the Fe–C–O bending mode. As may be seen from Table 3, the frequency of this mode is very sensitive to the type of axial ligand and has very little variation within a given type of ligand [also see Uno et al. (1987)]. Our value of 576 cm^{-1} for the CO-bound heme–HO complex confirms our assignment of a neutral imidazole.

The data from the ferric forms of the enzyme clearly demonstrate a transition from a six-coordinate high-spin configuration at neutral pH to a six-coordinate low-spin configuration under alkaline conditions. The identity of the sixth ligand in the complex at alkaline pH is proven to be a hydroxide from isotopic substitution. On the basis of the presence of the hydroxide ligand at alkaline pH and the similarity of the spectrum to that of myoglobin at neutral pH we infer that the axial ligand at neutral pH is a water molecule

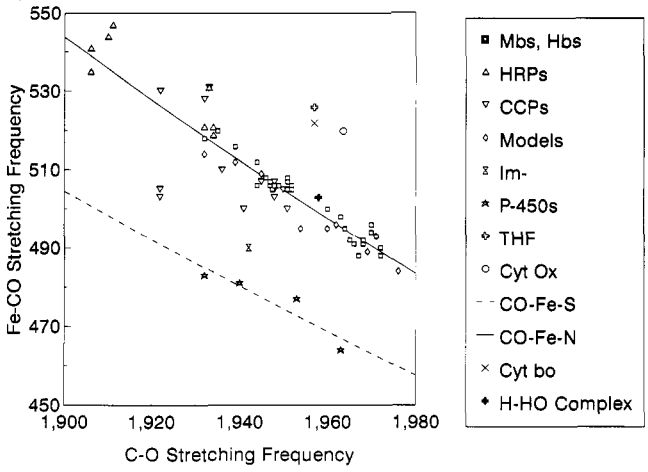

FIGURE 8: Correlation diagram of the Fe–CO stretching mode frequency and the C–O stretching mode frequency for a variety of heme proteins and porphyrin derivatives. Mbs, Hbs, HRPs, CCPs, and P-450s correspond to myoglobins, hemoglobins, horseradish peroxidases, cytochrome *c* peroxidases, and cytochrome P-450's, respectively. Models refers to porphyrin derivatives, Im- represents a porphyrin derivative with an imidazolate axial ligand. THF refers to a porphyrin derivative with tetrahydrofuran. Cyt Ox and Cyt bo refer to the mammalian cytochrome *c* oxidase terminal enzyme and the bacterial quinol terminal oxidase, respectively. The solid line is the correlation line when imidazole is the axial ligand, and the dashed line is the correlation when thiolate is the axial ligand. The point for the heme–heme oxygenase complex (•) lies near the imidazole correlation line.

Table 3: Fe–CO Stretching and Fe–C–O Bending Frequencies for Carboxy Heme Proteins with different Axial Ligands Coordinated to the Iron Atom[a]

proximal ligand	Fe–CO frequency (cm^{-1})	Fe–C–O frequency (cm^{-1})	protein	reference
His–Fe–CO	505–524	577–579	Mb, Hb, CcO	Tsubaki et al. (1982); Argade et al. (1984)
Im^{-}–Fe–CO	528–547	582–590	HRP, CcP	Uno et al. (1987); Smulevich et al. (1988)
Tyr^{-}–Fe–CO	542	593	catalase	Hu and Kincaid (1992)
S^{-}–Fe–CO	464–484	556–558	P-450	Uno et al. (1985)

[a] The bending mode is most sensitive to the type of coordinated ligand.

(Takahashi et al., 1994). To summarize, the axial ligands of the heme–HO complex are the same as those in myoglobin.

Effect of pH. While the pH change monitored by the high-frequency spectra of the complex is well explained by spin-state transitions, there are also several pH-induced changes observed in the low-frequency region. (1) In the 340–380-cm^{-1} region of the resonance Raman spectrum of the ferric complex, there are significant changes as a function of pH. The assignment of the 379-cm^{-1} line has not been established (Choi & Spiro, 1983; Lee et al., 1986), but it has been considered recently to be an out-of-plane vibrational mode which is coupled with axial ligand motions and/or motions of the peripheral substituents on the porphyrin ring (Sitter et al., 1988; Hu & Kincaid, 1993). Specifically, it has been found that this mode is sensitive to the propionic side chains (Hu & Spiro, 1993). We have already pointed out that the major changes result from the change in the spin equilibrium in the ferric complex. However, there are also small changes in the 340–380-cm^{-1} region for the reduced enzyme where there is no spin transition. These results indicate that there is a direct effect of the pH on the modes in this region. (2) The Fe–His stretching mode of the ferrous enzyme shows a shift to lower frequency by about 2 cm^{-1} in alkaline solution. This result is an additional indication that there is a conformational change associated with the pH transition which directly influences the porphyrin ring, even in the absence of exogenous ligands. Considering the fact that both the 379-cm^{-1} and Fe–His lines are assigned to out-of-plane modes, the change is probably along the axial direction of the heme plane. (3) No change is observed in the CO-bound ferrous form of the complex upon changing the solution pH. Since the Fe–CO stretching vibration is considered to be sensitive to the heme distal pocket environment, the insensitivity of the line indicates that the group which is responsible for the acid/base transition is not directly over the central iron atom. The qualitative observation that the CO-rebinding rate does not change with pH also suggests that the ionizable group does not lie along the pathway for ligand binding or escape.

The acid/base pH transition in ferric heme proteins is generally considered to be linked to a change in the protonation state of an amino acid residue in the distal pocket, although the identity of the residue is still speculative (Antonini & Brunori, 1971). Our observation of a pH-dependent frequency shift of the Fe–His stretching mode might suggest that changes in the proximal histidine are responsible for the acid/base transition. However, if the proximal histidine became deprotonated, a large shift to higher frequency would be expected rather than a small shift to lower frequency on the basis of the results from model compounds and horseradish peroxidase (Teraoka & Kitagawa, 1981). We propose that when the ionizable group, which is located near the heme but apparently not close to bound CO, is deprotonated, it causes a conformational change around the heme. This would affect the modes involving the propionates, which are involved in anchoring the heme to the heme pocket (Tomaro et al., 1984), causing the change in the mode at 379 cm^{-1}. In the ferrous ligand-free case this conformational change induces a heme reorientation resulting in the shift in the Fe–His stretching mode, which has been shown to be extremely sensitive in globins (Kitagawa, 1988). In the CO-bound form, the histidine–heme–CO linkages are stronger so that any conformational change is accommodated in other parts of the protein. Similar explanations have been put forth to account for the presence of large quaternary structure-dependent changes in the Fe–His stretching mode in deoxyhemoglobins and in the absence of any significant changes in the CO-bound forms (Rousseau et al., 1984). The absence of changes in the Fe–CO and C–O stretching modes is also consistent with the pH-independent behavior of CO-bound myoglobin (S. Takahashi and D. L. Rousseau, unpublished results).

Comparison of the Heme–Heme Oxygenase Complex to Myoglobin. The ligand structures of the heme–HO complex and myoglobin are the same, and their high-frequency Raman spectra coincide well with each other. The main difference between them appears in high-pH ferric proteins, where the heme–HO complex is primarily low spin while myoglobin is in a mixed-spin state. This shows the different heme pocket properties between these proteins, which are confirmed by the comparison of the low-frequency resonance Raman data. The largest changes appear as frequency changes and line broadenings of the modes which have out-of-plane or peripheral substituent character. This indicates that the conformations of ring substituents of the heme–HO complex are different from those of myoglobin and that there is a great deal of conformational freedom of the peripheral substituents in the the HO heme pocket. This is not an unexpected result since, in HO, the heme pocket must have sufficient flexibility to allow the heme to readily enter to form the enzyme-substrate complex and to leave as it becomes cleaved; and, on the other

hand, the pocket must have enough well-defined structure to control the specificity of the heme cleavage at the α-meso position of the heme. The necessity of the two propionic groups at the C6 and C7 sites of the heme for the HO activity has been demonstrated (Tomaro et al., 1984). While the heme is ligated in a way similar to that in myoglobin, the catalytic demands of the enzyme cause the interactions with the peripheral substituents to be quite different.

Catalytic Mechanism. It has been proposed that heme enzymes which catalyze the addition of oxygen to organic substrates or catalyze the oxidation of substrates by peroxides form an activated oxygen complex referred to as "compound I". This is an Fe^{4+}(ferryl)-oxo intermediate in which the heme is a π-cation radical and is both a good oxidant and a good oxo donor. Formation of a compound I type of intermediate is thought to proceed by what has been referred to as a "push–pull" mechanism (Ortiz de Montellano, 1986; Dawson, 1988; Poulos, 1988). In peroxidases, catalase, and cytochrome P-450's, the "push" is supplied by the anionic proximal ligands imidazolate, tyrosinate, and thiolate, respectively. Owing to the high electron density on these ligands, they are able to stabilize the high-valent oxidation states of the iron atom in the activated complex thereby facilitating cleavage of the O–O bond. On the distal side of the heme, charged residues contribute to the cleavage in the peroxidases by "pulling" on the O–O bond in the cleavage process. The heme–HO complex has neutral histidine as a fifth ligand, which contrasts to the push–pull class enzymes that have anionic axial ligands. Thus, its mechanism of oxygen activation may be very different from that in other oxygenases (Takahashi et al., 1994). It was proposed by Noguchi et al. (1983) that the reactive form of oxygen in the complex was a peroxo form rather than a ferryl form as in the P-450 type of catalysis. Recently, reaction of the enzyme complex with alkyl peroxides gave evidence which firmly ruled out a ferryl species as the hydoxylating agent and thereby supported a peroxo species as the activated form (Wilks & Ortiz de Montellano, 1993).

In consideration of the physiological function of the heme–heme oxygenase complex, a neutral imidazole may have advantages over imidazolate as the ligand bound at the fifth coordinate position. First, a great deal of structural flexibility of the protein must be necessary to allow binding of the heme and release of the catalytic products. The rigidity demanded by a strong proton-acceptor group near the histidine to form the imidazolate (Finzel et al., 1984) may not allow easy heme binding and release of ferrous iron. Second, the final step of the catalytic process is a reduction of ferric biliverdin to the ferrous form, which is then released from the enzyme (Yoshida & Kikuchi, 1978b). NADPH–P450 reductase, the physiological electron donor ($E_0' = -280$ mV; Vermilion & Coon, 1978), can catalyze this reduction, but ascorbate ($E_0' = 80$mV) cannot, indicating that the redox potential of ferric biliverdin complexed with HO lies between these two values and is the lowest among those of the several reduction steps of HO turnover (Yoshida & Kikuchi, 1978b). Since anionic ligand coordination is known to lower the redox potential of heme proteins (Poulus, 1988), the utilization of neutral histidine might be necessary for maintaining a sufficiently high redox potential for reduction by the reductase.

Conclusion. Resonance Raman spectroscopy of the heme–HO complex was used to characterize the coordination structure and properties of the complex. The complex has a neutral histidine as an axial ligand, which places it in a very small class of heme-containing monooxygenases, dioxygenases, and peroxidases as previously noted (Takahashi et al., 1994). Indeed, only one other monooxygenase, *Pseudomonas aminovorans* secondary amine monooxygenase (SAMO), having a neutral imidazole as the axial ligand has been reported (Alberta et al., 1989), but its catalytic mechanism is yet to be determined. However, the monooxygenase mechanism of heme oxygenase may be unique due to the unusual property of the enzyme of the heme serving as both the catalytically active cofactor and the enzyme substrate. The acid/base pH transition of the heme–HO complex is associated with a conformational change around the heme. The low pK_a of the transition (7.6), detected in the ferric form of the enzyme complex, suggests the presence of an ionizable group that gives up its proton near neutral pH. This may be important physiologically by allowing the facile protonation of the bound oxygen in the formation of a peroxide intermediate. Further characterization of the structure and reaction mechanism of the heme–HO complex may help clarify the functional mechanisms of the other oxygenases with a neutral imidazole and will enrich our understanding of biological oxygen activation chemistry.

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