Pentacoordinate Hemin Derivatives in Sodium Dodecyl Sulfate Micelles: Model Systems for the Assignment of the Fifth Ligand in Ferric Heme Proteins

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ABSTRACT Ferric iron protoporhyrin IX derivatives in SDS micelles have been investigated by means of visible absorption, resonance Raman, and XANES spectroscopies to establish specific correlations between the marker bands of the pentacoordinate derivatives obtained from the three different techniques. Hydroxyl and 1,2-dimethyl imidazole coordinated hemins display the typical spectroscopic marker bands of a pentacoordinate high-spin ferric iron derivative in both Raman and XANES spectra. In turn, the optical absorption spectra of these two derivatives are very different. This difference is in line with the assignment of hydroxyl as the fifth coordination ligand to free hemin in SDS micelles, as demonstrated by the isotopic shift of the frequency of Fe-OH bond with $H_2{}^{18}O$. The present assignments are relevant to the identification of the coordination state and the nature of the fifth ligand in ferric heme proteins.

INTRODUCTION

The understanding of the mechanisms of ligand binding to heme proteins relies on the assignment of the heme iron coordination states. This information is crucial to the identification and characterization of the thermodynamic and kinetic barriers encountered by the incoming ligand. In ferrous heme protein derivatives, the coordination state of the iron atom has been assigned unequivocally by means of a variety of spectroscopic techniques. The analysis of simple optical absorption spectra suffices to establish the coordination state of ferrous iron. Hence, in hemoglobins and myoglobins, the liganded and unliganded species can be identified in static and transient measurements of both the visible and Soret optical absorption bands (Antonini and Brunori, 1971). The situation is radically different in the ferric state, at least for the high-spin iron complexes. Although ferric heme proteins are amenable to a number of spectroscopic investigations that are sensitive to specific stereochemical features of the heme environment, a definite answer on both the coordination state and the nature of the fifth ligand still requires a combination of techniques. In fact, the analysis of the visible absorption spectra of ferric high-spin hemoglobin does not allow an unequivocal distinction between pentacoordinate or hexacoordinate species, particularly as far as the presence or absence of a hydroxyl or water molecule in the sixth coordination position is concerned.

Absorption spectra of pentacoordinated heme proteins have been reported to display broad high-spin marker bands centered at either 600–605 or 630 nm together with low values of the molar absorptivity in the Soret region around 395–405 nm (Ikeda-Saito et al., 1992). However, these absorption bands overlap with those typical of high-spin hexacoordinate derivatives. In some cases the optical absorption spectra of water or hydroxyl hexacoordinate derivatives and proximal histidine pentacoordinate derivatives appear to be essentially indistinguishable (Maltempo, 1976). In several heme proteins investigated to date, a further difficulty is due to the presence of equilibrium mixtures of five- and six-coordinate derivatives in which a water molecule provides the sixth ligand (Giacometti et al., 1981; Boffi et al., 1994). The use of EPR spectroscopy may furnish additional information on the nature of the ferric pentacoordinate species. EPR spectra of ferric pentacoordinate heme proteins display a multiplicity of signals in the high-spin region. In several proteins, a splitting of the $g = 6$ signal coupled to the presence of lower spin species has been reported, suggesting the presence of a quantum spin admixture of $S = 3/2$ and $S = 5/2$ in pentacoordinate derivatives (Maltempo, 1976; Ikeda-Saito et al., 1992; Boffi et al., 1994; Bogumil et al., 1995). Resonance Raman spectroscopy provides unique information on the effective occupancy of the axial coordination positions through the identification of so-called spin-state marker bands (Hu et al., 1996; Wang et al., 1996). However, the frequency of these bands is ligand insensitive, and the nature of the axial ligand can be assessed only through isotope dependence studies. In recent years, the development of x-ray absorption techniques has furnished new, powerful methods for a detailed analysis of the environment of the iron atom in terms of site symmetry and bond distances and angles. Nevertheless, the assignment of marker bands relative to the coordination state of ferric iron still relies on very few experimental observations (Ikeda-Saito et al., 1992).

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The simplest possible model system that mimics the heme environment in a heme protein consists of hemin dissolved in a detergent solution over its critical micellar phase (Simplicio, 1972; Mazumdar and Mitra, 1993). In these systems, the heme is embedded in the micelles, thus simulating the hydrophobic cavity of heme proteins and, as in heme proteins, it can coordinate a variety of ligands. Moreover, imidazole-based ligands can be coordinated to the iron atom to simulate the proximal His coordination, in both the ferric and ferrous states (Hori and Kitagawa, 1980). In the present paper, visible absorption, resonance Raman, and XANES spectroscopies have been used in combination on the same set of ferric heme derivatives. By means of this approach, specific differences between hydroxyl- and imidazole-based pentacoordinate ferric heme can be singled out that are of relevance for the assignment of these species in heme proteins. Specific examples are discussed that call for a reexamination of extensive prior data.

MATERIALS AND METHODS

Hemin in SDS micelles was prepared by adding a few microliters of a hemin (Sigma Aldrich Co., St. Louis, MO) solution in 0.1 M NaOH to 10 ml of a 0.2 M sodium phosphate buffer solution containing 10% SDS. Under these conditions, hemin is monodispersed in the SDS micelles (Simplicio, 1972), and its concentration is ~20–60 μM. The bis-imidazolate ferric derivative was prepared by adding an excess of solid imidazole to a final concentration of 0.25 M. The ferric bis-imidazolate complex was assigned a molar absorptivity at 542 nm of 11,200 M^{-1} cm^{-1} by comparing its absorption spectrum with that obtained on the same solution with the pyridine hemochromogen method (Falk, 1975). The ferric pentacoordinate derivative with 1,2-dimethyl-imidazole was obtained by adding the solid substance to the hemin-SDS solution to a final concentration of ~0.1 M. Absorption spectra were measured on a Jasco V560 spectrophotometer (Jasco, Tokyo, Japan).

The Fe K-edge x-ray spectra were collected in the fluorescence mode at the beam line D21 of the LURE synchrotron facility, by using an energy-resolving array detector, made by seven elements of hyperpure Ge from Canberra Industries. The energy resolution at the Fe K_α fluorescence (6400 eV) was 170 eV. A Si(311) channel-cut single crystal was used as the monochromator. The spectral resolution at the Fe K-edge is ~1 eV, and energy shifts of resolved absorption peaks of 0.5 eV can be detected. Harmonic contamination was rejected by using a total reflection mirror after the monochromator. The spectra had a total signal averaging of 14 s/point. The fluorescence count jumped from 70 c/s/element before the edge (7100 eV), to ~650 c/s/element above the edge (7250 eV), giving a total count jump of ~56,000. In all experimental spectra presented here, the energy is aligned at the absorption threshold of metallic Fe foil.

In resonance Raman measurements, an incident laser frequency of 413.1 nm (Kr-ion laser; Spectra Physics, Mountain View, CA) was used, and the Raman scattered light was dispersed through a polychromator (Spex, Metuchen, NJ) equipped with a 1200 grooves/mm grating and detected by a liquid nitrogen-cooled CCD camera (Princeton Instruments, Princeton, NJ), as described elsewhere in detail (Wang et al., 1996). The samples were placed in a spinning cylindrical cell with a 2-mm light path. A holographic notch filter (Kaiser, Ann Arbor, MI) was used to remove the laser scattering. Typically six 30-s spectra were recorded and averaged after removal of cosmic ray spikes by a standard software routine (CSMA; Princeton Instruments). The concentration of heme used for the Raman measurements was ~150 μM in 200 mM sodium phosphate buffer. The following final compositions were used in isotopic water: 150 μM ferric hemin, 10% SDS, 200 mM sodium phosphate buffer (pH 8.3), ~80% $H_2{}^{18}O$ or D_2O (using 98% $H_2{}^{18}O$ from Cambridge Isotope Laboratories, Andover, MA; 99.9% D_2O from Aldrich, Milwaukee, WI), and ~20% $H_2{}^{16}O$.

RESULTS

The absorption spectra in the visible and Soret regions of different ferric heme derivatives in SDS micelles are shown in Fig. 1. The absorption spectrum of the low-spin, bis-imidazole complex resembles that reported in the literature and displays the characteristic features of ferric imidazole hemeprotein complexes (Antonini and Brunori 1971; Simplicio, 1972), i.e., a band centered at 536 nm with a shoulder at 562 nm and a peak at 415 nm, in the visible and Soret regions, respectively (Fig. 1 *A*). The absorption spectrum of the 1,2-dimethyl-imidazole pentacoordinate adduct is characterized by a typical "high-spin marker band" centered at ~600 nm and by a low value of the molar absorptivity at 400 nm (Fig. 1 *B*). In the hemin solution without exogenous ligands (free hemin), the high-spin marker band is sharper and more intense than that observed in the imidazole pentacoordinate derivative and is shifted to 605 nm (Fig. 1 *C*).

The resonance Raman spectra of bis-imidazole, 1,2-dimethyl-imidazole complexes, and free hemin in SDS micelles are shown in Fig. 2, in the region between 1300 and 1700 cm^{-1}, where the oxidation-, coordination-, and spin-state marker bands occur. In particular, the spectral regions around 1460–1510 cm^{-1} (ν_3), 1520–1590 cm^{-1} (ν_2), and 1600–1650 cm^{-1} (vinyl stretch and ν_{10}) are known to be

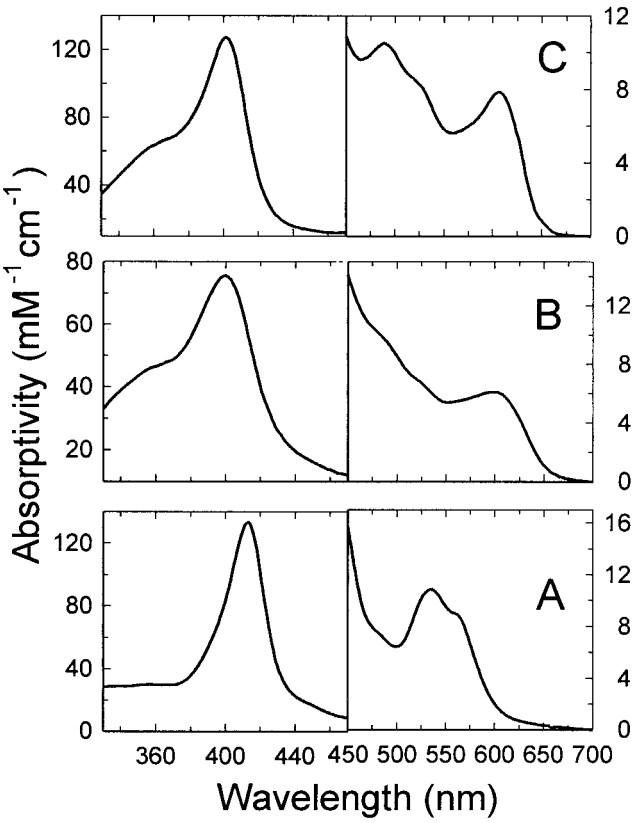

FIGURE 1 Absorption spectra of hemin complexes in SDS micelles in the visible and Soret regions. (*A*) Hemin bis-imidazole complex. (*B*) Hemin 1,2-dimethyl imidazole complex. (*C*) Free hemin. All hemin solutions were in 200 mM phosphate buffer, 10% SDS.

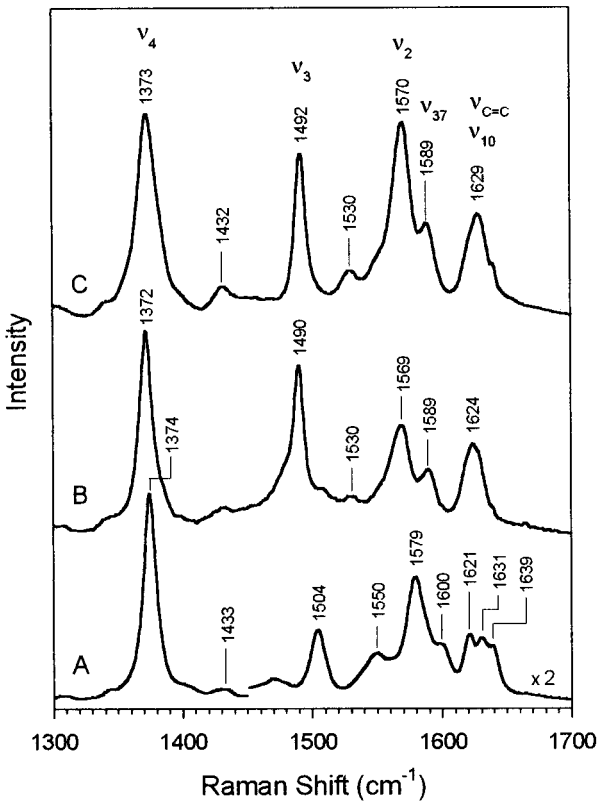

FIGURE 2 Resonance Raman spectra of the ferric hemin complexes in SDS micelle in the high-frequency region. Spectra shown are (*A*) hemin bis-imidazole complex, (*B*) hemin 1,2-dimethyl imidazole complex, and (*C*) free hemin, in sodium phosphate buffer, pH 8.3, 100% $H_2{}^{16}O$. The laser excitation wavelength was 413.1 nm, and the power delivered to the sample was ~10 mW.

sensitive to the iron coordination and spin state of the heme iron (Wang et al., 1996). Thus the resonance Raman spectra in Fig. 2 can be analyzed by the peak frequencies of the ν_2, ν_3, and ν_{10} modes. The spectrum of the bis-imidazole complex (Fig. 2 *A*), as expected, is a clear hexacoordinate low-spin species, as demonstrated by the peak position of ν_3 at 1504 cm^{-1} and ν_2 at 1570 cm^{-1}. In turn, the spectra of both the free hemin and of the 1,2-dimethyl-imidazole complexes (Fig. 2, *B* and *C*) show distinctive features of high-spin pentacoordinate derivatives, namely ν_3 at ~1490 cm^{-1} and ν_{10} at ~1570 cm^{-1}. The two spectra are very similar in terms of the peak frequencies of different marker bands. Only small shifts (within 2 cm^{-1}) are observed for the iron core size marker band (ν_4) as well as for other bands in the 1400–1600 cm^{-1} frequency region. A more sizable (5 cm^{-1}) frequency shift is observed for the vinyl stretching band ($\nu_{C=C}$) at 1624 cm^{-1} for the alkaline hemin with respect to 1629 cm^{-1} for the 1,2-dimethyl-imidazole derivative.

The assignment of hemin in the absence of exogenous ligands as a pentacoordinate species was unexpected, because free hemin in micelles had been proposed to be either a bis-aquo or a mono-hydroxyl/mono-water hexacoordinate species, based on different spectroscopic techniques (Simplicio, 1972). To identify the fifth coordination ligand, the isotopic shift for the iron-oxygen stretching mode (Fe-OH) has been searched for and detected in experiments carried out with free hemin in $H_2{}^{16}O$ and $H_2{}^{18}O$. Fig. 3 shows the low-frequency region of the resonance Raman spectra of ferric hemin in SDS micelles in buffered solutions (pH 8.3) of water of various isotopic compositions. Small differences are detected in the 520–580 cm^{-1} region in the comparison between $H_2{}^{18}O$ (*spectrum c*) relative to $H_2{}^{16}O$ (*spectrum a*). The other features in the two spectra remain virtually unchanged. The presence of some porphyrin modes in the 550–570 cm^{-1} region made it difficult to determine the exact frequency of the isotope-sensitive lines in the absolute spectra. However, the sensitivity of the Raman modes to water isotopes could be readily observed in the difference spectrum (*spectrum e*). We assign the line at ~577 cm^{-1} with the oxygen isotope sensitivity as the iron-hydroxide stretching mode ($\nu_{Fe\text{-}OH}$) arising from the Fe-OH moiety of the five-coordinate heme in SDS micelles. The difference bands seen at the ~350 cm^{-1} region arise because of a small difference (0.1–0.2 cm^{-1}) in frequency of the intense 351 cm^{-1} band, and these are viewed as insignificant. The spectrum in D_2O (*spectrum b*) does not change appreciably

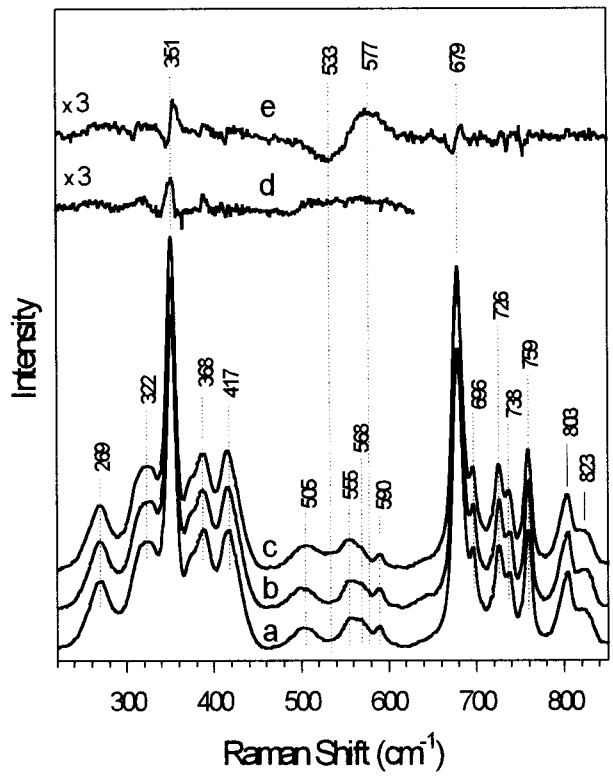

FIGURE 3 Resonance Raman spectra of free hemin in the low-frequency region. The concentration of heme was in 150 μM in 200 mM sodium phosphate buffer, 10% SDS, pH 8.3. Spectra shown are in (*a*) 100% $H_2{}^{16}O$, (*b*) 80% D_2O and 20% $H_2{}^{16}O$, (*c*) 80% $H_2{}^{18}O$ and 20% $H_2{}^{16}O$, (*d*) spectrum *a* minus spectrum *b*, and (*e*) spectrum *a* minus spectrum *c*. The laser excitation wavelength was 413.1 nm, and the power delivered to the sample was ~10 mW.

compared to that in H_2O, as also seen from the difference spectrum (*spectrum d*).

The Fe K-edge XANES spectra of bisimidazole ferric hemin, 1,2-dimethyl-imidazole-coordinated, and free hemin adducts are shown in Fig. 4 (*left*) together with their derivative spectra (*right*) from top to bottom, respectively. Spectra 2 and 3 can be assigned to pentacoordinate high-spin species on the basis of the following considerations: 1) the intensity of peak C_1 at 7122 eV is lower in pentacoordinate derivatives with respect to hexacoordinate ones. A lower intensity of the C_1 peak, according to multiple scattering theory (Durham, 1988), is due to a lower number of collinear multiple scattering pathways with respect to octahedral complexes, 2) the pre-edge peak P at 7111 eV is enhanced in pentacoordinate derivatives with respect to hexacoordinate derivatives. Pre-edge peaks observed in K-edge XANES spectra have been interpreted according to the coordination symmetry of the metal complexes. A pre-edge peak observed for tetrahedral transition metal clusters of the type MO_4 was assigned to a dipole allowed transition from the A_1 ground state to an unoccupied anti-bonding T_2 orbital (Kutzler et al., 1980). The peak intensity depends on the electron density of the molecular d orbitals, because the final state has d symmetry. Thus the peak is absent from absorbing metals with filled d shells (e.g., Cu(I) $3d_{10}$) and is very strong for $3d_0$ compounds. In octahedral, centrosymmetrical clusters, very small pre-edge peaks are referred to as $A_{1g} \rightarrow T_{2g}$ or $A_{1g} \rightarrow E_g$ dipole forbidden transitions. For noncentrosymmetrical clusters, the peaks increase in intensity because of metal p-d orbital mixing. Other mechanisms have been reported to contribute to intensity, e.g., metal 3d orbital mixing, magnetic dipole and electric quadrupole transitions, and vibronic coupling with the metal-ligand vibrations (Sano et al., 1992). Although the main mechanisms are not yet fully understood, the pre-edge peak is always observed to be enhanced in pentacoordinate with respect to hexacoordinate hemeproteins (Oyanagy et al., 1987; Shiro et al., 1990; Ikeda-Saito et al., 1992). The spectra in Fig. 4 clearly show that the intensity of peak C_1 decreases, whereas peak P is enhanced in both hydroxyl coordinated and 1,2-dimethyl-imidazole hemin complexes, in agreement with literature data reporting XANES changes from hexacoordinate to pentacoordinate hemeproteins (Ikeda-Saito et al., 1992).

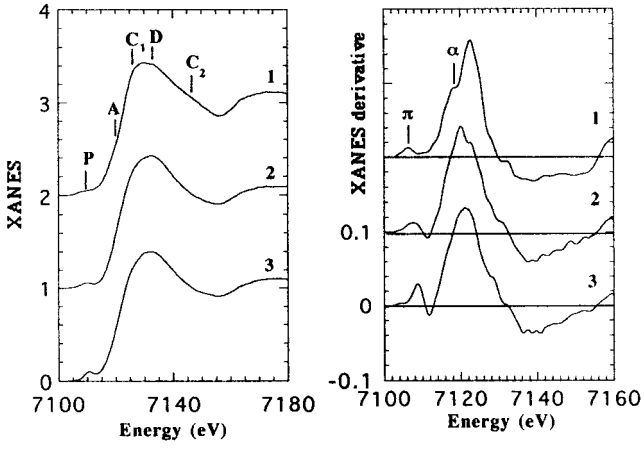

FIGURE 4 XANES spectra of hemin complexes in SDS micelles. (*Left, from top to bottom*) Hemin bis-imidazole complex, hemin 1,2-dimethyl imidazole complex, free hemin. (*Right*) First derivative spectra relative to the compounds in the left panel. The heme concentration was 1–2 mM in 200 mM sodium phosphate buffer, 10% SDS, pH 8.3.

DISCUSSION

The determination of the ferric iron coordination state and the nature of the bound ligands is a central problem in porphyrin biochemistry that can be addressed by a variety of spectroscopic methods. A comment on the spectroscopic markers in the assignment of the iron coordination state is in order, as different spectroscopies allow the monitoring of different stereochemical features.

The resonance Raman spectra of a large number of heme proteins and model compounds have demonstrated that the frequencies of several lines are sensitive to the coordination and spin state of the central iron atom of the heme. The nature of the in-plane skeletal stretching vibrational modes corresponding to the lines between 1450 and 1640 cm^{-1} has been elucidated by normal mode calculations (Spiro, 1975; Abe et al., 1978; Spiro and Li, 1988). Experimentally, most of these modes have been observed to display a linear inverse correlation with the porphyrin core size (Spiro and Li, 1988). The core size depends on both the coordination number and the spin state (Spiro and Li, 1988). The frequency dependence of the vibrational modes on the core size was inferred to originate from changes in the force constants of the methine bridge bonds as the porphyrin ring expands or contracts, because the pyrrole rings are so rigid. Therefore, within the same spin state, in comparing a pentacoordinate species to a hexacoordinate species, the iron atom is expected to move out of plane, resulting in a contraction of the porphyrin cavity and a shortening of the methine bridge bonds, with a concomitant increase in frequency of the modes with methine bridge contributions. However, in addition to the effect of the coordination, the effect of the occupancy of the dx^2-y^2 orbital must also be considered. The presence of an electron in this orbital in the high-spin configuration causes lengthening of Fe-pyrrole nitrogen bonds, thus rendering the porphyrin core expanded, leading to a decrease in the stretching frequencies of the relevant modes (ν_2, ν_3, and ν_{10}). In the hexacoordinate high-spin complexes, the porphyrin ring is expanded the most as the iron atom resides in the porphyrin plane an because the dx^2-y^2 orbital contains an electron. Thus, in hexacoordinate high-spin complexes, the stretching frequencies of the core size marker bands are lowest, whereas in the hexacoordinate low-spin species where there is no population of the dx^2-y^2 orbital, the core is the smallest and the frequencies of the vibrational modes are the highest. In summary, the frequency of the core-size-dependent bands

increases in the following order: hexacoordinate high spin < pentacoordinate high spin < hexacoordinate low spin. In this framework, the resonance Raman spectra of the complexes in Fig. 2, *B* and *C*, are clearly assigned as pentacoordinate species.

The XANES spectra provide a different view of the coordination state of the ferric high-spin heme iron. In particular, the "pre-edge" P peak intensity appears to reflect the "d-orbitals" symmetry and their electron density. 1s → 3d transitions are dipole forbidden in hexacoordinate octahedral systems but gain intensity upon distortion of the ligand symmetry (p-d orbital mixing) and with a decrease in the iron 3d electron density. Within the approximation that describes ligand binding to the iron atom as an electron donation from the ligand σ orbital, the intensity of the P peak reflects the sum of two contributions, namely the degree of electron donation to the iron atom from the axial ligands and the degree of mixing of the iron p-d orbitals.

Optical absorption spectroscopy in the visible and Soret regions monitors the symmetry of the porphyrin π-electron distribution as well as the presence of iron-porphyrin charge transfer bands. These spectral features are not uniquely related to the coordination state of the heme iron. In particular, if the symmetry of the pentacoordinate derivative departs from the expected C_{4V} space group, the visible spectrum is also expected to undergo significant changes. Nevertheless, a criterion for the assignment of the five coordinates can be established based on the matching between the observed spectrum of the heme protein and those of the pentacoordinate heme model compounds reported here.

Hydroxyl coordinated hemin and 1,2-dimethyl imidazole hemin adducts in SDS micelles are characterized by markedly different absorption spectra in the visible region (Fig. 1), although their Raman and XANES spectra are very similar and clearly assign these compounds as pentacoordinate derivatives. In particular, the hydroxyl coordinated hemin displays an absorption spectrum characterized by an intense charge transfer band at 605 nm, whereas the 1,2-dimethyl imidazole adduct shows a broad and less intense band at 600 nm. In the absence of exogenous imidazole ligands, the heme is not a hexacoordinate (bis-aquo) complex (Simplicio, 1972), but a pentacoordinate, hydroxyl-coordinated species, as demonstrated by the assignment of the coordination from the high-frequency resonance Raman spectrum and by the detection of the iron oxygen stretching band in the low-frequency region of the spectrum. Hence, a ligand such as a hydroxyl group destabilizes the binding of a second weak ligand molecule (i.e., water) in the *trans* position favoring the formation of a pentacoordinate derivative. Destabilization of the weak axial ligand in the *trans* position is known to occur upon binding of a single molecule of a strong σ-donor ligand such as cyanide, imidazole, or pyridine to iron porphyrins and phthalocyanins dissolved in weakly coordinating solvents such as water or dimethyl sulfoxide (Boffi et al., 1998). In these solvents, therefore, the monoligated species with a strong σ-donor is effectively a five-coordinated one in which the *trans* bond with the weakly coordinating solvent molecule has been disrupted. In turn, evidence of *trans* ligand destabilization with weak σ-donors (Cl^-, F^-) is limited to the occurrence of five coordination upon anion binding in apolar, noncoordinating solvents (dichloromethane or carbon tetrachloride), where the dominant force that stabilizes the complex is the electrostatic interaction between the anion and the charged iron atom, rather than σ donation (ionic couple formation) (Spiro and Burke, 1976).

The present findings have important consequences for the assignment of the iron coordination chemistry of hemeproteins. In fact, in a number of hemoglobins and myoglobins, the ferric pentacoordinate species has been implicitly identified as a proximal "histidine bound" species, an assumption that is challenged by the present data. A convincing example is provided by the ferric homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis*, in which a pentacoordinate heme iron is the dominant species at alkaline pH values (Boffi et al., 1994). Under these conditions the absorption spectrum is characterized by an intense high-spin marker band at 605 nm and is almost superimposable on the spectrum of hydroxyl-coordinated hemin in SDS micelles. On this basis, contrary to the previous tentative assignment as a proximal histidine pentacoordinate species, the ferric *Scapharca* hemoglobin pentacoordinate species is a hydroxyl "five-coordinate" complex in which the bond to the proximal histidine is absent (Das et al., 1999a). In contrast, the visible absorption spectrum of *Aplysia limacina* Mb at acidic pH values has been demonstrated to be predominantly a pentacoordinate, proximal histidine-bound ferric derivative (Giacometti et al., 1981). However, this spectrum is similar to the absorption spectrum of the 1,2-dimethyl imidazole adduct in that the high-spin marker band is at 600 nm and is broad and less intense. Thus the visible absorption spectrum can provide important clues to the nature of the fifth ligand, which, however, must be confirmed by resonance Raman and/or XANES spectroscopy. It should be stressed that the visible absorption spectra of imidazole-based pentacoordinate ferric derivatives may display small but significant changes in the peak position and in the intensity of the high spin marker bands, as observed in different proximal-His pentacoordinate myoglobin mutants (Ikeda-Saito et al., 1992). The visible absorption spectrum of the 1,2-dimethyl-imidazole heme adduct in micelles clearly refers to a "constrained" heme conformation with a slightly distorted C_{4v} symmetry. Thus changes in the local symmetry of the heme imposed by the protein moiety may lead to significant alteration of the absorption spectrum with respect to the model system.

It is worth noting that the nature of the isotope shift of the Fe-OH stretching frequency reported thus far in micellar heme systems is different from those observed in ferric hemeproteins. Thus, in alkaline ferric hemoglobin and myoglobin, which are hexacoordinate heme complexes with a proximal histidine bond, the frequency of $\nu_{Fe\text{-}OH}$ is observed in the range of 490–560 cm^{-1} (Asher and Schuster, 1979;

Feis et al., 1994). The isotope shifts observed in both proteins are nearly the ideal values of an isolated Fe-OH harmonic oscillator. In the case of pentacoordinate hydroxide heme complexes such as the H93G myoglobin mutant (Das et al., 1999b) and *Scapharca inaequivalvis* HbI (Das et al., 1999a), likewise, the observed isotope shifts are very close to the expected values ($\sim$24 cm^{-1} in H_2O versus $H_2{}^{18}O$). In contrast, for the heme in micelles reported here, the difference spectrum ($H_2{}^{16}O$ versus $H_2{}^{18}O$) shows a maximum and a minimum at 577 and 533 cm^{-1}, respectively, i.e., a difference of 44 cm^{-1}. Such a large shift in the frequency of $\nu_{Fe\text{-}OH}$ in $H_2{}^{18}O$ is not anticipated, as the isotope shift ($^{16}O/^{18}O$) of $\nu_{Fe\text{-}OH}$ frequency would ideally be 23.8 cm^{-1}, assuming that the two oscillating units are the Fe and the OH and that they form an "isolated" Fe-OH harmonic oscillator. Hence it is very likely that the bandwidth (FWHM) of the $\nu_{Fe\text{-}OH}$ line is much larger in the present case relative to those observed in protein environments ($\sim$20 cm^{-1}). A small difference between two wide bands (actual separation in frequency $<$ bandwidth) would give rise to an anomalous difference spectrum (Rousseau, 1981). In Fig. 5 we present a simulation of the difference spectrum in which the width of the Fe-OH stretching mode was minimized to obtain a best fit to the data. In this simulation, the width was found to be $\sim$60 cm^{-1}. Observation of such a broad $Fe\text{-}OH^{stretching}$ band has also been observed in a ferric pentacoordinate iron-porphyrin complex in which the $\nu_{Fe\text{-}OH}$ frequency was observed at 541 cm^{-1} in aqueous solution and the width was $\sim$65 cm^{-1} (Reed et al., 1990). The observation of a much broader $\nu_{Fe\text{-}OH}$ line in heme-micellar system is very interesting in that it suggests a heterogeneity of the environment experienced by the Fe-OH moiety. A plausible situation that would explain how such heterogeneity arises is conformational flexibility of the Fe-O-H moiety. This is not very surprising, as porphyrins are known to be very dynamic in nature inside micelles (Maiti et al., 1995). Hence the Fe-O-H group in a protein environment is conformationally more restricted than in micelles. We postulate that the reason no significant isotope shift was observed in D_2O is that the Fe-O-H unit has a bent structure such that the motion of the proton (or deuteron) is not coupled with the motion of the oxygen in the Fe-O stretching mode. In such a situation, the Fe-O-H oscillator would show an isotope shift in $H_2{}^{18}O$, but would not be responsive to replacement of the hydrogen by deuterium (in D_2O).

In conclusion, the occurrence of "five coordination" in ferric hemeproteins should not be uniquely associated with the presence of the proximal histidine as the iron ligand. Five coordination to a distal hydroxyl molecule may occur in many different heme proteins.

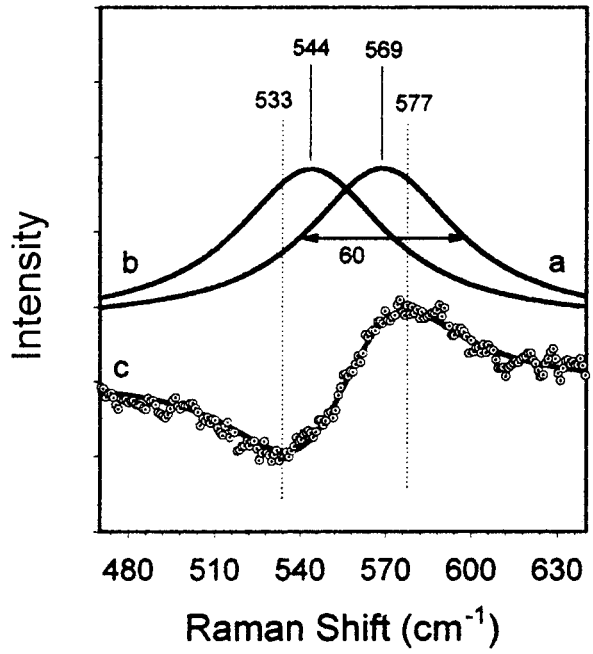

FIGURE 5 Simulation of Raman difference spectrum ($H_2{}^{16}O - H_2{}^{18}O$) of the Fe-OH stretching mode. The frequency difference between the two isotopically shifted bands (spectrum *a* for $H_2{}^{16}O$; spectrum *b* for $H_2{}^{18}O$) was set at 25 cm^{-1}. The peak position and the widths of the two bands were varied uniformly to obtain the best fit (*solid line* through spectrum *c*) to the difference spectrum (shown as *open circles*, spectrum *c*) from the data reported in Fig. 3, spectrum *e*. A width of $\sim$60 cm^{-1} for the bands located at 569 and 544 cm^{-1} yielded the best fit to the difference spectrum, with the maxima and minima at 577 and 533 cm^{-1}, respectively.

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