Hydroxide Rather Than Histidine Is Coordinated to the Heme in Five-coordinate Ferric *Scapharca inaequivalvis* Hemoglobin*

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The ferric form of the homodimeric *Scapharca* hemoglobin undergoes a pH-dependent spin transition of the heme iron. The transition can also be modulated by the presence of salt. From our earlier studies it was shown that three distinct species are populated in the pH range 6–9. At acidic pH, a low-spin six-coordinate structure predominates. At neutral and at alkaline pHs, in addition to a small population of a hexacoordinate high-spin species, a pentacoordinate species is significantly populated. Isotope difference spectra clearly show that the heme group in the latter species has a hydroxide ligand and thereby is not coordinated by the proximal histidine. The stretching frequency of the Fe-OH moiety is 578 cm^{-1} and shifts to 553 cm^{-1} in H$_2$^{18}O, as would be expected for a Fe-OH unit. On the other hand, the ferrous form of the protein shows substantial stability over a wide pH range. These observations suggest that *Scapharca* hemoglobin has a unique heme structure that undergoes substantial redox-dependent rearrangements that stabilize the Fe-proximal histidine bond in the functional deoxy form of the protein but not in the ferric form.

The homodimeric hemoglobin (HbI)[1] isolated from arcid clam *Scapharca inaequivalvis* possesses unique structural features (1–14). Although HbI has a low sequence homology with tetrameric mammalian hemoglobins (Hb), it has a conserved globin fold. It displays a unique structural basis for cooperative ligand binding in that the two hemes face each other across the intersubunit contact and are able to communicate directly through their propionate groups (2, 4, 15, 16). As a consequence of this unusual structural linkage, cooperativity is accompanied by major tertiary changes in the heme environment with only minor rearrangements of the quaternary structure, in contrast to mammalian Hbs (2, 13). The subunit interface in HbI is formed mostly by the helices E and F, which are solvent-exposed in tetrameric mammalian Hbs.

Although a wealth of structural and functional information is available on the ferrous deoxy and ligand-bound forms of HbI

(2–14), little is known about the oxidized form (1). The heme structure in the ferric form undergoes a spin transition that is dependent on pH and salt concentration (17, 18). From our earlier studies by optical and resonance Raman spectroscopy, it was shown that a mixture of three distinct species are populated in the pH range 6–9 (17, 18). The formation of a six-coordinate low-spin heme is favored at acidic pH and high ionic strength and is accompanied by the reversible dissociation of the HbI dimer into monomers. This species is likely to be formed by coordination of the distal histidine to the heme (in addition to the proximal histidine) as shown by EPR studies (18). At neutral pH values, a dimeric pentacoordinate species appears and becomes the dominant form at alkaline pH and low salt. A population of dimeric six-coordinate high-spin heme exists over the entire 6–9 pH-range and in both low and high salt. This six-coordinate high-spin species is a typical aquomet form (18) such as that commonly observed in mammalian myoglobins (Mb) and hemoglobins (19, 20).

The occurrence of a low-spin bishistidine heme has been proposed to exist in some invertebrate Hbs (21–23). However, conversion of a hexacoordinate species into a pentacoordinate form, as seen in HbI, is very rare in Mbs and Hbs, and the structure of the pentacoordinate species in HbI is not known. The ferric Mb from the mollusk *Aplysia limacina* is also reported to contain a pentacoordinate species at acidic pH (5.9) in which the proximal histidine is proposed to serve as the fifth ligand (24). The observation of a strong pH-dependence of the ferric form of *Aplysia* Mb was suggested to be the consequence, in part, of the absence of a distal histidine (24). *Scapharca* HbI, on the other hand, contains a distal histidine yet also shows a strong pH dependence of the heme ligation structure. The optical spectrum and the relative intensity of the Raman marker line (ν_3) of the pentacoordinate species in *Aplysia* Mb, however, are very different from the pentacoordinate species seen in *Scapharca* HbI. Furthermore, as described above, the nature of the pH-dependent transition in *Scapharca* HbI is very unique as compared with the aquo-hydroxy transition in vertebrate Mbs and Hbs. Hence, it is important to determine whether the unique optical properties reflect some unanticipated axial ligation states in ferric *Scapharca* HbI.

Studies on the ferric form of Hbs (metHb) have significantly advanced our understanding of the structural basis for the cooperative transition in Hb. Under physiological conditions, metHb is formed by spontaneous autoxidation, and the redox equilibrium is shifted toward the reduced form by the metHb reductase system. Recently it was shown that the formation of metHb may result also from interaction with NO *in vivo* (25). In addition, metHbs are extremely useful to study because numerous ligands form stable ferric heme complexes that have a wide range of electronic properties. Therefore, several studies on metHbs have been carried out as a means of understanding the mechanism of allosteric control (26–33).

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[1] The abbreviations used are: HbI, homodimeric hemoglobin; HbA, human adult hemoglobin; Hb, hemoglobin; Mb, myoglobin; metHb, ferric hemoglobin; MES, 4-morpholineethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
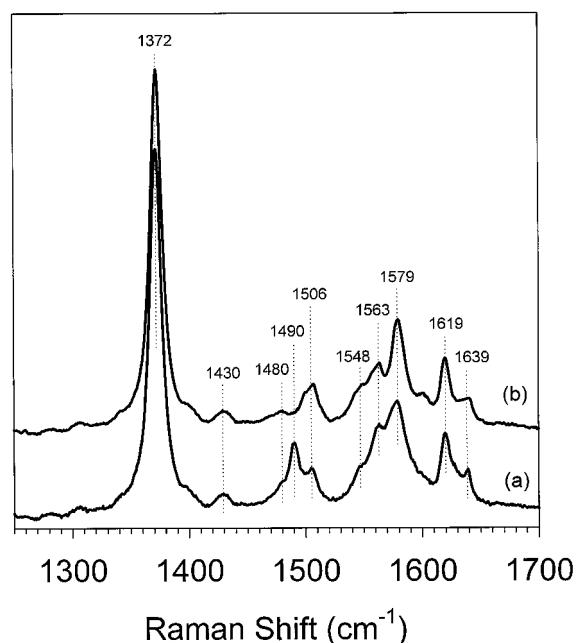
FIG. 1. **Resonance Raman spectra of the ferric form of the** *Scapharca* **HbI in the high frequency region.** Spectra shown are in 100% $H_2^{16}O$, pH 9.0 (*a*) and pH 6.0 (*b*). Laser excitation wavelength was 413.1 nm, and power delivered at sample was 8 mW.
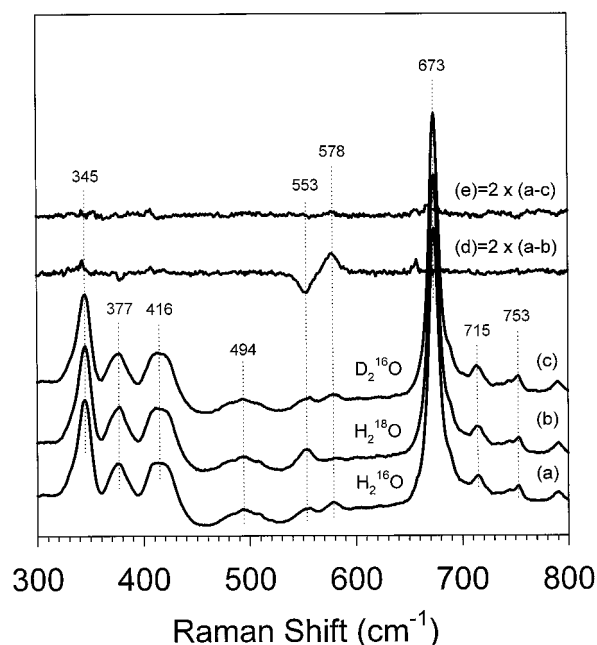

FIG. 2. **Resonance Raman spectra of the ferric form of the** *Scapharca* **HbI in the low frequency region.** Protein concentration was 35 μM, in 40 mM CHES buffer, pH 9.0. Spectra shown are in 100% $H_2^{16}O$ (*a*), 80% $H_2^{18}O$ and 20% $H_2^{16}O$ (*b*), 80% D_2O and 20% $H_2^{16}O$ (*c*), *spectrum a* minus *spectrum b* (*d*), *spectrum a* minus *spectrum c* (*e*). Other experimental conditions are the same as in Fig. 1.

In the present study, we have characterized the pentacoordinate form of oxidized *Scapharca* HbI by resonance Raman spectroscopy and identified the fifth ligand to the heme unambiguously as hydroxide rather than histidine. This finding, which provides direct evidence for cleavage of the Fe-proximal His bond in ferric HbI, is discussed in the framework of known manifestations of strain in that bond in ferrous HbI and in liganded T-state human hemoglobin crystals (34).

EXPERIMENTAL PROCEDURES

The HbI from *S. inaequivalvis* was isolated and purified as described elsewhere (5). The protein samples were oxidized by addition of potassium nitrite to the oxygenated protein. The excess oxidant was removed by gel filtration through a Sephadex G-25 column equilibrated with the desired buffer. The protein was stored in liquid nitrogen until use.

The concentration of the protein samples used for the Raman measurements was 35 μM in 40 mM buffer (MES, pH 6.0; phosphate, pH 7.4; CHES, pH 9.0). To prepare the protein samples in isotopic water, the following composition was used: 35 μM ferric HbI, 40 mM buffer, 80% $H_2^{18}O$ or D_2O (using 98% $H_2^{18}O$ from Cambridge Isotope Laboratories, Andover, MA; D_2O from Aldrich, Milwaukee, WI), and 20% $H_2^{16}O$. Deoxy samples were prepared by adding an aliquot of dithionite solution to an anaerobic HbI solution prepared in buffers at the desired pH values. Absorption spectra were recorded before and after the Raman measurements to ensure the stability of the species studied. The samples were placed in a spinning cylindrical cell with a 2-mm light path. An incident laser frequency of 413.1 nm (Kr-ion laser, Spectra Physics) 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 charge-coupled device camera (Princeton Instruments, Princeton, NJ) as described elsewhere in detail (35). A holographic notch filter (Kaiser, Ann Arbor, MI) was used to remove the laser scattering. Typically, five 30-s spectra were recorded and averaged after removal of cosmic ray spikes by a standard software routine (CSMA, Princeton Instruments, NJ).

RESULTS

The resonance Raman spectra of ferric *Scapharca* HbI, shown in Fig. 1, were measured at acid (pH 6.0) and alkaline (pH 9.0) pHs in 40 mM buffer. At alkaline pH, the pentacoordinate heme species is very prominent as judged by its ν_3 band at 1490 cm^{-1}. The spectrum shows also the presence of a six-coordinate low-spin (ν_3 = 1506 cm^{-1}) species and a small population of six-coordinate high-spin (ν_3 = 1480 cm^{-1}) species. At acid pH, however, the low-spin species is dominant.

To identify the nature of the pentacoordinate species, the low frequency region of the spectra were measured at alkaline pH as a function of the isotopic composition of the water. The low frequency region of the resonance Raman spectrum is very useful in identifying metal-ligand vibrations as it can directly demonstrate the presence of a particular ligand and the nature of its interactions in the heme pocket (35). In particular, a line in the 490–560 cm^{-1} region has been assigned to the Fe-OH stretching frequency in alkaline ferric hemoglobin, myoglobin, and horseradish peroxidase (19, 20). The position of this line is sensitive to the spin state of the metal and the strength of hydrogen bonding to the hydroxide moiety of these proteins. Fig. 2 shows the low frequency region of the resonance Raman spectra of ferric *Scapharca* HbI in alkaline-buffered solutions of water of various isotopic composition. The Raman line at 578 cm^{-1} in $H_2^{16}O$ (Fig. 2, *spectrum a*) shifts to 553 cm^{-1} in $H_2^{18}O$ (*spectrum b*), and yields a clear difference spectrum (*spectrum d*). However, the spectrum in D_2O (*spectrum c*) does not change appreciably compared with that in H_2O as also seen from the difference spectrum shown in *spectrum e*. We assign the line with the oxygen isotope sensitivity as the iron-hydroxide stretching mode ($\nu_{\text{Fe-OH}}$) arising from the Fe-OH moiety of the five-coordinate heme in HbI. As expected for this assignment, the feature at 578/553 cm^{-1} is absent at pH 6 (data not shown), where the population of the five-coordinate heme is negligible. No isotope sensitivity was observed for any other line in the spectra at pH 6.

It is important to note that the 25 cm^{-1} isotope shift (^{16}O/^{18}O) corresponds to a nearly ideal value (23.8 cm^{-1} assuming that the two oscillating units are the Fe and the OH) that would be expected from an "isolated" Fe-OH harmonic oscillator. In the event the OH group had strong hydrogen-bonding or strong non-bonding interactions, the expected isotope shift would not be observed because of a deviation from a perfect two-body oscillator (19). One intriguing observation is that the
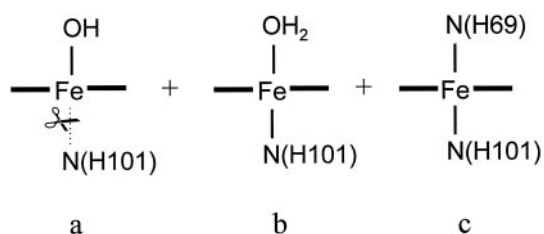
FIG. 3. **A scheme depicting the heme structure of various species in ferric *Scapharca* HbI in the pH range 6–9.** *a*, a five-coordinate high-spin species that has a hydroxide ligand, and the proximal histidine (His-101) bond is broken. This species dominates at neutral and alkaline pH. *b*, a six-coordinate high-spin aquomet species that has a bound water (or a high-spin hydroxide) with an intact proximal histidine. This species is seen at low levels over the entire pH range studied. *c*, a six-coordinate low-spin species that has the distal histidine (His-69) and proximal histidine as axial ligands. This species becomes the major form at acidic pH.

Fe-OH moiety did not show any appreciable D_2O effect. We postulate that such a situation could arise if the Fe-O-H unit exists in a significantly bent configuration so that the vibration of the O-H unit is not coupled with that of the Fe-O unit. In such a case, 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).

DISCUSSION

In summary, the three heme species identified by optical and resonance Raman spectroscopy can be schematically represented as shown in Fig. 3. The pentacoordinate high-spin species (Fig. 3a) is unambiguously assigned in the present work as a hydroxide form. Therefore, the proximal histidine bond is no longer retained; instead, the hydroxyl group binds to the iron accounting for the fifth ligand. The species shown in Fig. 3, *b* and *c* are, respectively, the high- and low-spin hexacoordinated forms, both of which retain their proximal Fe-His bond, but have water and the distal histidine, respectively, as their sixth ligands. The identity of the high-spin hexacoordinate form (Fig. 3b) is based on the similarity of its optical and resonance Raman spectra to those of aquomet Mbs and Hbs. The assignment of the low-spin hexacoordinate form (Fig. 3c) relies on our previous EPR study (18) that determined *g*-values similar to those of the bishistidine heme complexes. Hence, it was inferred that the distal histidine binds the heme iron at acidic pH.

In six-coordinate hydroxy complexes of hemeproteins with histidine or imidazole as an axial ligand, the ν_{Fe-OH} mode is located in the 450–560-cm^{-1} region (19, 20, 36). In the five-coordinate form of HbI, ν_{Fe-OH} appears at a significantly higher frequency. We attribute the high frequency to two origins. First, the absence of a ligand trans to the hydroxide can strengthen the Fe-OH bond in comparison with the case when the proximal histidine is present. Second, the higher frequency of the line as compared with the five-coordinate hydroxide species in the H175G mutant of cytochrome *c* peroxidase (37), as well as it being significantly narrower, indicates that there are no strong interactions between it and any neighboring residues in its binding pocket. The similarity of the very high frequency for ν_{Fe-OH} in the hydroxide-bound heme in HbI to that in H93G Mb, also shown to be a five-coordinate hydroxide complex,[2] suggest that the hydroxide group has a hydrophobic environment and there is a partially collapsed structure of the proximal cavity in HbI just as in H93G Mb. Thus, on cleavage of its bonding to the heme iron, the proximal histidine under-

goes a substantial structural rearrangement resulting in a partial collapse of the proximal cavity. In turn, the heme, which remains inside the heme pocket because of stabilization by hydrophobic interactions, has an altered orientation as indicated by the changes in the polarized absorption spectra of ferric HbI crystals brought to alkaline pH (38).

In contrast to ferric HbI, the ferrous form remains quite stable over a wide pH range, as judged from the invariance of all its properties, including the resonance Raman spectrum (data not shown). One specific feature is of interest, namely the very low Fe-His stretching frequency (203 cm^{-1}) of the deoxygenated protein, which indicates a high degree of proximal strain (39). Despite the proximal strain, the protein is able to bind oxygen cooperatively even if that leads to movement of the iron into the heme plane presumably straining the Fe-histidine bond even further. However, ligand-binding to the ferrous protein is facilitated by movement of Phe-97 that swings out of contact with the heme and the proximal histidine on ligand binding in the ferrous protein, hence performing a dual task of information transfer to the contralateral subunit and partially reducing the extra strain on the proximal histidine. We postulate that such structural rearrangements characteristic of the deoxy ↔ oxy transition do not take place in the hydroxide form in the ferric protein. If binding hydroxyl leaves the protein in its "T-like" state, Phe-97 does not reorient and will cause steric crowding of the proximal histidine destabilizing the Fe-His bond. The aftermath of the Fe-His bond rupture is a partial collapse of the proximal cavity that retains the heme group as a pentacoordinate hydroxide-bound species.

Observation of a pentacoordinate ferric heme in native myoglobins or hemoglobins is uncommon (19, 34, 40). A pentacoordinate species observed in the *Aplysia* Mb at acidic pH values has a much broader and less intense ν_3 line at 1495 cm^{-1}, which is suggested to arise because of the destabilization of water binding to the heme and not to the breakage of the proximal histidine (24). In the proximal-histidine mutant of sperm whale Mb (H93G) on the other hand, the ν_3 line appears at 1490 cm^{-1}. In the absence of the proximal histidine, H93G stabilizes the heme as a pentacoordinate hydroxide-bound species[2]. Horseradish peroxidase stabilizes a pentacoordinate heme but without cleaving the proximal bond. Similarly, in freshly prepared native cytochrome *c* peroxidase, a five-coordinate heme is the stable form, coordinated by the proximal histidine (41). In a mutant (H175G) of cytochrome *c* peroxidase, where the proximal histidine was mutated to a non-coordinating ligand, the heme showed a mixture of aquomet complexes including a pentacoordinate species that was suggested to be a heme-hydroxide complex (37).

The cleavage of the Fe-proximal His bond in alkaline ferric HbI is particularly intriguing in light of recent crystallographic observations on human cyano-met T-state ferric HbA (34). Quite unexpectedly, the analysis of the heme geometry and coordination state revealed that the distance between the iron and the nitrogen of the proximal histidine in the cyanide-bound α-chains is much too long to allow for covalent bond formation (2.9–3.1 Å). Thus, the α-chains in ferric, cyanide-bound T-state HbA is another example of a pentacoordinate species in which the proximal bond is broken because of the presence of a strong iron ligand on the distal side. Yet, in the β chains, cyanide binding leaves the proximal bond unaltered, indicating that its effective strength does not depend solely on the presence of a strong distal ligand. Moreover, in contrast to the behavior of the hydroxide adduct, in the cyano complex of HbI, there is no evidence for rupture of the proximal histidine bond (12). In this connection, it should be noted that a number of spectroscopic markers point to a strained, distorted coordination of the heme

iron in ferrous HbI, *e.g.* the broad anisotropic EPR signal of the Co-porphyrin protein (42), the small hyperfine shift of the N_δ proton resonance (43) and the unusually low frequency of the Fe-His mode (39). Interestingly, these features are shared by the α chains in the ferrous HbA tetramer (44, 45). Thus, the heme coordination geometry and the properties of the exogenous ligand both determine whether or not the proximal histidine bond will be ruptured.

In several ferrous heme proteins, rupture of the proximal histidine bond has been observed when NO adducts are formed (13, 46–51). In deoxy hemoglobin, a five-coordinate NO complex forms in the α-chains in the presence of inositol hexaphosphate, and in myoglobin, the five-coordinate NO complex forms at low pH (pH ~4). The five-coordinate form of ferrous guanylate cyclase, generated by the coordination of NO, has been postulated as the active structure that catalyzes the formation of cyclic GMP (47, 49–51). Thus, determination of the factors that lead to the rupture of the proximal histidine bond are essential for the full understanding of the functional forms of these proteins.

In conclusion, the present data on HbI and the comparison with liganded T-state HbA suggest that pentacoordinate derivatives which lack the proximal iron-histidine bond do not occur solely in selected hemoglobin and myoglobin mutants but may have a general significance in the native proteins. The biochemical consequences of this unique heme coordination on the kinetics and thermodynamics of anionic ligand binding to ferric hemoproteins remain to be determined and will be the object of future investigations.

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