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Structural Characterization of Oxidized Dimeric *Scapharca inaequivalvis* Hemoglobin by Resonance Raman Spectroscopy*

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Resonance Raman spectra of the ferric homodimeric hemoglobin from *Scapharca inaequivalvis* have been measured over the pH range 5.8–8.3 in buffers of ionic strengths 0.01 and 0.1 M to determine the spin and coordination state of the iron atom. Three species contribute to the spectra: a low spin hexacoordinate, a high spin pentacoordinate, and a high spin hexacoordinate component. Optical absorption and EPR spectra measured under the same conditions allowed the identification of the ligands in the sixth coordination position, namely the distal histidine in the low spin derivative and a water molecule in the high spin one. The relative concentrations of these three species depend on pH in an unusual way. Thus, the aquomet derivative is present over the whole pH range, albeit in small amounts as most of the hemoglobin converts to the low spin hemichrome at acid pH values and to the pentacoordinate derivative at neutral and slightly alkaline ones. The formation of a pentacoordinate heme as the pH is increased has not been reported previously for other myoglobins and hemoglobins. Low ionic strength and high protein concentration favor the formation of the high spin pentacoordinate species, while at high ionic strength and low protein concentration the low spin hexacoordinate species prevails. Ionization of the iron-bound water molecule occurs at pH ≥ 9.3; accordingly, signals from the hydroxyl derivative were not observed in the Raman spectra over the pH range studied.

The homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis* (HbI) has been the topic of many investigations in recent years in view of its unique structural and functional properties (1). The globin chains have the classical myoglobin fold, but are assembled through the heme-carrying E and F helices, which in vertebrate hemoglobins are exposed to solvent. This unique mode of assembly endows the protein with high cooperativity in oxygen binding (n = 1.5) as it permits direct information transfer between the two heme groups. At variance with vertebrate hemoglobins, oxygenation does not entail major quaternary rearrangements of the subunits, but is accompanied only by marked tertiary changes in the heme environment (2, 3). In accordance with these characteristics, the subunit interface is stable toward dissociation into monomers to a similar extent in oxy- and deoxy-HbI. In fact, neither of the derivatives shows significant dissociation into monomers in the pH range 6–9, at high salt or low protein concentrations (4).

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In contrast to the stability of the ferrous derivatives, the ferric protein undergoes a pH-dependent dissociation into monomers that is more marked at acid pH values and is accompanied by a high spin to low spin transition as indicated by parallel optical absorption and sedimentation velocity experiments (5). The low spin species has been identified as a hemichrome based on its optical absorption bands at 526 and 560 nm (5). No specific heme environment was assigned to the dimeric high spin species, characterized by the concomitant presence of absorption bands at 600 and 630 nm, the former prevailing at alkaline pH values and the latter at acid ones (5), and by the occurrence of an axial to rhombic transition which manifests itself in EPR experiments at alkaline pH and has been attributed to the onset of a constraint at the heme site (6).

The present resonance Raman study was undertaken to characterize the heme environment of ferric HbI at different pH values and thereby obtain information on the tertiary changes in the heme pocket that lead to the instability of the quaternary structure. Extensive resonance Raman studies on heme model compounds and hemoproteins have allowed for the identification of specific Raman lines that are sensitive to the spin state of the iron atom, to its coordination, and to the size of the porphyrin core (7–13).

MATERIALS AND METHODS

S. inaequivalvis HbI was extracted and purified as described in Ref. 4. Oxidation was obtained by addition of solid potassium nitrite to the oxygenated protein. The excess oxidant was removed by gel filtration on a Sephadex G-25 column equilibrated with the desired buffer (0.1 or 0.01 M MOPS[1]-NaOH was used in all experiments). The fluoride and cyanide derivatives were obtained by adding solid NaF or NaCN to the oxidized protein until the absorption spectra did not change upon further salt addition.

Resonance Raman spectra were measured by directing a 50-milliwatt beam at 413.1 nm on the rotating samples (14). The scattered light was dispersed by a single polychromator equipped with a 1200 groove/nm grating and detected by a cryogenically cooled CCD camera. The spectral slit width was 5 cm^{-1}. The Rayleigh scattering was removed from the scattered light with a holographic filter. Optical absorption spectra were measured on a Cary 3 spectrophotometer at 20 °C. EPR measurements were carried out on a Bruker ESP 300 spectrometer at liquid helium temperature.

RESULTS

The resonance Raman spectra of ferric HbI were measured at 5 pH values in the range 5.8–8.3 under conditions which stabilize either the high spin, dimeric species or the low spin, monomeric one, namely high protein concentration and 0.01 M ionic strength or low protein concentration and 0.1 M ionic strength. The spectra are reported in Figs. 1*A* and 2*A*, respectively. Under both conditions, significant changes occur as a function of pH in the 1470–1525 cm^{-1} region (ν_3), as well as in the 1550–1600 cm^{-1} (ν_2) and in the 1620–1640 cm^{-1} (vinyl stretch and ν_{10}) regions.

[1] The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.

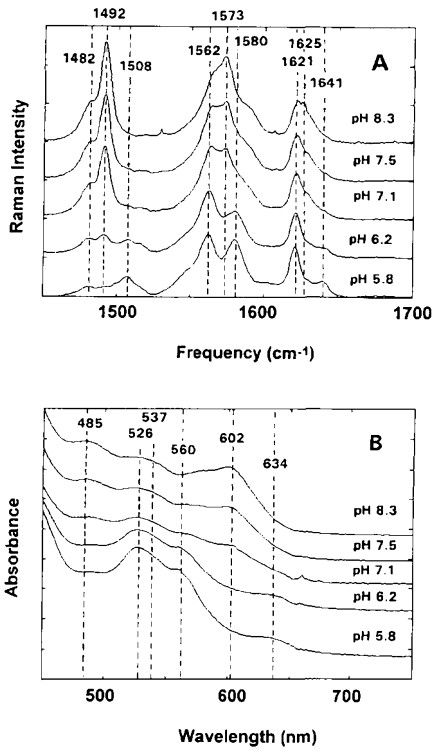

FIG. 1. **Resonance Raman (*A*) and optical absorption (*B*) spectra of ferric HbI as a function of pH in 0.01 M MOPS-NaOH buffer.** Protein concentration, 0.4 mM. Laser excitation wavelength, 413.1 nm; power, 50 milliwatts.

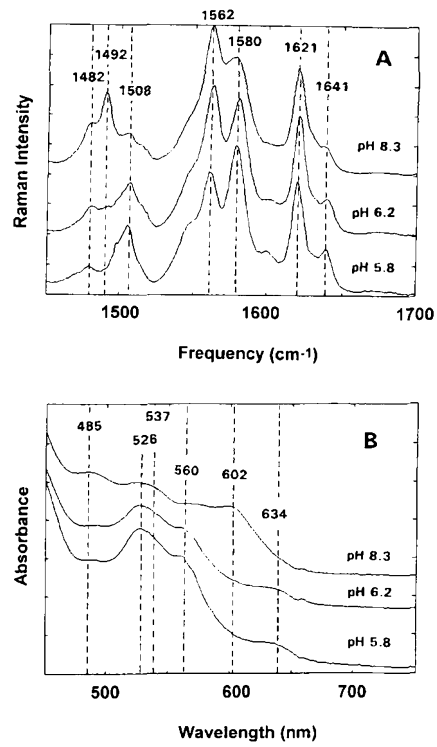

FIG. 2. **Resonance Raman (*A*) and optical absorption (*B*) spectra of ferric HbI as a function of pH in 0.1 M MOPS-NaOH buffer.** Protein concentration, 0.1 mM. Laser excitation wavelength, 413.1 nm; power, 50 milliwatts.

These regions are known to be sensitive to both the coordination and spin state of the iron (7–10, 13).

In 0.01 M ionic strength buffers at alkaline pH (Fig. 1*A*), ν_3 displays a major line at 1492 cm^{-1} and a shoulder at 1482 cm^{-1}. Upon decreasing pH, the intensity of the 1492 cm^{-1} line decreases markedly and a peak at 1508 cm^{-1} appears. In the ν_3 region, three peaks are observed at 1562, 1573, and 1580 cm^{-1}. The first and the last prevail at low pH values, while the intermediate peak is the dominant species at pH 8.3. The ν_{10} region is characterized by the presence of a line at 1621 cm^{-1} throughout the entire pH range. In addition, under alkaline conditions, there is a broad line with a maximum at about 1625 cm^{-1}. As the pH is lowered, this broad line decreases in intensity and a well resolved line at 1641 cm^{-1} develops.

In 0.1 M ionic strength buffers (Fig. 2*A*), the lines occur at the same frequencies described for 0.01 M ionic strength, but are characterized by different relative intensities.

The optical absorption spectra, measured in parallel with the Raman ones at the two ionic strengths are shown in Figs. 1*B* and 2*B*. At acid pH values, the absorption spectrum is characterized by the presence of a main peak at 526 nm with shoulders at 485 and 560 nm and a further peak at 634 nm. At alkaline pH values, the spectra display four main peaks at 485, 537, 580, and 602 nm. Therefore, upon lowering the pH, the most conspicuous changes are the large decrease of the 602 nm band and the concomitant appearance of the peak at 634 nm, as already reported by Spagnuolo *et al.* (5). At the higher ionic strength studied similar changes occur, but are of smaller extent.

Reference Raman spectra of high and low spin adducts of ferric HbI have been obtained with the fluoride and cyanide derivatives (Fig. 3*A*). In fluoride HbI, ν_3 appears as a single peak at about 1480 cm^{-1}, and ν_2 is centered around 1560 cm^{-1}. In the ν_{10} region, only a single line at 1620 cm^{-1} was detected. In cyanide HbI, ν_3 is at 1508 cm^{-1}, ν_2 at 1580 cm^{-1}, and ν_{10} at 1640 cm^{-1}. The corresponding optical absorption spectra (Fig. 3*B*) are characteristic of high and low spin derivatives (15).

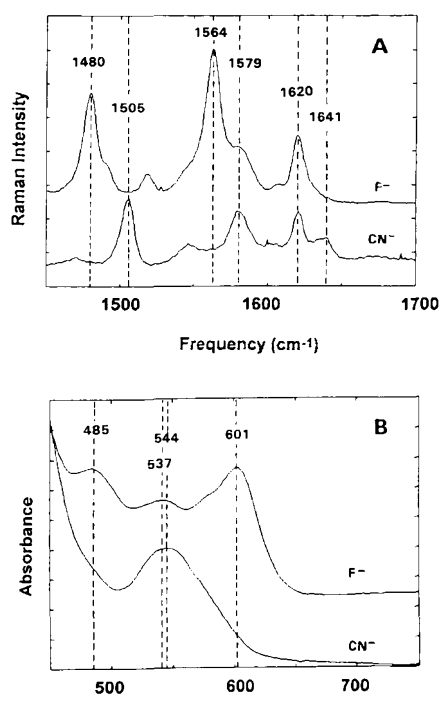

FIG. 3. **Resonance Raman (*A*) and optical absorption (*B*) spectra of fluoride and cyanide ferric HbI.** Protein concentration, 0.4 mM in 0.1 MOPS-NaOH buffer, pH 7.0.

EPR spectra were measured at 5K as a function of pH in 0.01 M and 0.1 M buffers. At pH 6.5, in accordance with the optical absorption spectra given in Figs. 2*B* and 3*B*, a strong low spin signal is detected at $g = 3.4$ (Fig. 4, *A* and *B*) in addition to an axial high spin signal at $g = 6.0$. At pH values higher than 7.0

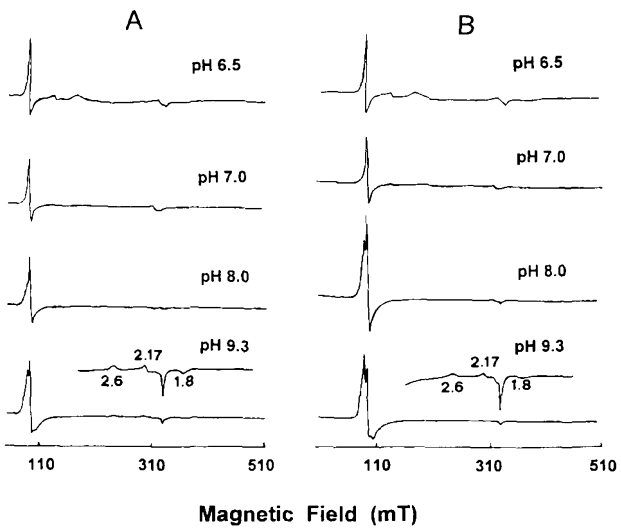

FIG. 4. **EPR spectra of ferric HbI as a function of pH.** Buffer: 0.1 M (*A*) and 0.01 M (*B*) MOPS-NaOH buffer, pH 7.0.

at both ionic strengths, the spectrum is characterized by the appearance of a rhombic high spin signal (g_1 = 6.18, g_2 = 2.60, g_3 = 1.8) which becomes more evident with increase in pH. At pH 9.3, a small percentage of a low spin species is detected at g_1 = 2.60, g_2 = 2.17, g_3 = 1.80.

DISCUSSION

The resonance Raman spectra of a number of ferric hemoproteins and model compounds have brought out the empirical sensitivity of the vibrational modes in the high frequency region of the porphyrin macrocycle to the iron coordination and spin state (7–10). This sensitivity results from the normal coordinates associated with the modes in this region. These modes involve primarily C_α-C_m and C_α-C_β stretching vibrations which are sensitive to the structure in the porphyrin center (17). Thus, the frequencies of the modes in this region correlate with the porphyrin core size, more specifically $d(C_t - N)$, the distance between the center of the porphyrin core and the pyrrole nitrogens (9, 16). The iron coordination and spin affect the core size and thereby yield the configuration-sensitive characteristic frequencies. Although these empirical correlations are established for ν_3, ν_2, and ν_{10}, other modes in the high frequency region which overlap with these modes make their use as quantitative determinants of spin and coordination states difficult. This is especially true of ν_2 which overlaps with ν_{11}, ν_{19}, ν_{37}, and ν_{28} as well as of ν_{10} which overlaps with the ever present vinyl stretching mode at about 1620 cm^{-1} (9, 10).

In general, for hexacoordinate Fe(III) hemes, the frequencies of the spin-sensitive lines are higher for the low spin than for the high spin derivatives. For pentacoordinate Fe(III) hemes, the frequencies are intermediate between these two extremes and depend on the degree of non-planarity of the heme (9). Thus, it is possible to assess the iron coordination and spin state for any given hemoprotein.

As expected on the basis of these considerations, fluoride and cyanide HbI (Fig. 3*A*) display resonance Raman spectra typical of high spin and low spin hexacoordinate hemes, respectively. In fluoride HbI, ν_3 is a single peak at about 1480 cm^{-1}, ν_2 is centered at about 1560, and ν_{10} appears to overlap with the vinyl mode at about 1620 cm^{-1}. Conversely, in the spectrum of cyanide HbI, ν_3 occurs at 1508 cm^{-1}, ν_2 at 1580 cm^{-1}, and ν_{10} at 1640 cm^{-1}. The frequencies of the relevant peaks are identical within a few cm^{-1} to those reported for the corresponding HbA and Mb derivatives (9, 12).

The resonance Raman spectra of ferric HbI at 0.01 M ionic strength and the pH-induced changes in the range 5.8–8.3 (Fig. 1*A*) can be interpreted as follows. A hexacoordinate high spin heme is present throughout the whole pH range as shown by the peaks at 1482 and 1562 cm^{-1}. At acid pH values, the additional presence of a hexacoordinate low spin heme is indicated by the low spin marker frequencies at 1508, 1580, and 1641 cm^{-1}. With an increase in pH, these lines decrease sharply in intensity and can no longer be detected at neutral and alkaline pH values, where the spectrum displays strong peaks at 1492 cm^{-1} (ν_3) and at 1573 cm^{-1} (ν_2), characteristic of pentacoordinate hemes. The increase in their intensity with the increase in pH is paralleled by a change in the opposite direction of the low spin marker bands.

Therefore, the pattern of heme coordination and spin state of ferric HbI emerging from the analysis of the Raman data is quite clear. At 0.01 M ionic strength, a 6-coordinate high spin heme is present in small amounts over the whole pH range studied; at acid pH values, the major species is represented by a low spin hexacoordinate heme, whereas a pentacoordinate, high spin heme prevails in the neutral and alkaline range. The increase in ionic strength to 0.1 M alters the relative proportions of the species (Fig. 2*A*). At any given pH value, the amount of the low spin component is enhanced with respect to the high spin pentacoordinate component, while the high spin hexacoordinate species is essentially unaffected.

The conversion of a hexacoordinate heme to a pentacoordinate heme as the pH is increased has not been observed previously in other myoglobins or hemoglobins. Although the quaternary structures of ferrous and ferric HbI must differ in many respects (see accompanying paper (23)), we postulate that the proximal site of pentacoordinate and hexacoordinate species in the ferric protein differs in much the same way as in the corresponding ferrous derivatives. In the ligand-free form of the ferrous protein, Phe^{97} packs up against the heme and the proximal histidine and thereby exerts a strain on the iron-histidine bond. In turn, this strain is reflected in the resonance Raman spectra in which the iron-histidine stretching mode appears at 203 cm^{-1}, a value lower than that reported in any other heme protein (18). Upon coordination of CO, and presumably O_2, Phe^{97} swings away from the heme to allow for the necessary stereochemical changes associated with the binding of the ligand. In the oxidized protein, the stability of the pentacoordinate form of the met-protein is higher than that of the hexacoordinate forms at alkaline conditions indicating that the change from the strained to the unstrained structure is promoted by a protonation step.

The EPR experiments provide additional focus in that they add information on the nature of the ligands in the sixth coordination position. At pH 6.5, the axial high spin signal at g = 6 is ascribable to an aquomet-heme (19). In turn, the broad g = 3.4 signal which dominates the low field spectrum is due most likely to coordination of the distal histidine, which gives rise to a highly anisotropic low spin complex, although coordination of Met^{37} cannot be ruled out completely (20). At the other extreme of pH, the small signal at g_1 = 2.6, g_2 = 2.17 and g_3 = 1.80 points to the presence of an iron-bound hydroxyl species (Fig. 4, *C* and *F*). In turn, these findings indicate that the p*K* of the acid/alkaline transition of the heme-bound water molecule is ≥9.3. Accordingly, no signal originating from the iron-OH stretching mode has been detected in resonance Raman experiments performed in D_2O in the pH range 5.8–8.3 (data not shown). At intermediate pH values, the axial signal displays a clear rhombic distortion (g values of 6.18, 2.60, and 1.8) which becomes more evident with increases in pH. In a previous paper (6), this signal had been attributed to the onset of a constraint at the

heme site. However, based on the resonance Raman data, the rhombic signal can be assigned to the pentacoordinate heme. This assignment is supported by the similarity of the EPR and resonance Raman spectra of ferric HbI and those of other pentacoordinate hemoproteins, *e.g.* ferricytochrome *c'* at pH $\geq$ 10 (21) and the a_3^{+3}-Cu_{a3}^{+2}-NO complex of cytochrome oxidase (22).

The optical absorption spectra (Figs. 1*B* and 2*B*) are fully consistent with the resonance Raman and EPR data. At acid pH values, the HbI spectrum can be accounted for in terms of a low spin hemichrome (absorption bands at 526 and 560 nm) and small amounts of a high spin, aquomet derivative (absorption maximum at 485 and 634 nm). Moreover, the pentacoordinate derivative can be correlated with the high spin peak at 602 nm, typical of weak field ligands, which characterizes the spectra at pH values $\geq$ 7.0. In accordance with the resonance Raman data, the optical absorption experiments indicate that the increase in ionic strength to 0.1 M favors the formation of the low spin hemichrome.

In conclusion, the present study has allowed the identification of three distinct heme environments in ferric HbI over the pH range 5.8–8.3: a low spin bis-imidazole, in which the distal histidine occupies the sixth coordination position, and two high spin forms, an aquomet and a pentacoordinate derivative. At pH values $\geq$ 9.3, a fourth component, namely the hydroxyl derivative, is formed. Thus, the distinguishing features of HbI are on the one hand the relative instability of the aquomet derivative, which forms the low spin hemichrome at acid pH values and the pentacoordinate derivative at neutral and slightly alkaline ones, and on the other the unusually high pK of the acid/alkaline aquomet transition. The mechanism which leads to the interconversion of the different species and relates them to the monomerization equilibrium is intriguing. This aspect is addressed in the accompanying paper (23) in which a modification of the previously proposed scheme (5) will be presented to account for all the new observations.

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