Protein–Heme Interactions in Hemoglobin from the Mollusc *Scapharca inaequivalvis*: Evidence from Resonance Raman Scattering[†]

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ABSTRACT: Resonance Raman spectra of the *Scapharca inaequivalvis* homodimeric hemoglobin (HbI) have been measured for the ligand-bound and ligand-free ferrous forms of the protein. In the deoxy derivative, the iron–histidine (Fe–His) stretching mode, proposed as a marker of the oxygen affinity and a conduit linking the hemes to the subunit interface, gives rise to a Raman peak centered at 203 cm^{-1}, an unusually low frequency compared to that reported for other hemoglobins and myoglobins. In the CO-bound derivative, three isotope-sensitive lines at 517, 583, and 1945 cm^{-1} have been assigned to the Fe–CO stretching, Fe–C–O bending, and C–O stretching modes, respectively. From the frequencies of these modes and from their relative intensities, the Fe–C–O geometry appears to be tilted from axial coordination and shows a bending angle which has been estimated to be about 171 ± 5°. For the oxygen derivative, only one isotope-sensitive peak has been detected at 570 cm^{-1}, in line with the values found for myoglobin and other hemoglobins. Resonance Raman spectra of HbI modified with *p*-(chloromercuri)benzoate (PMB) at Cys^{92} have been measured in parallel with those of the native protein. Despite the large increase in oxygen affinity produced by the PMB modification, the frequency of the Fe–His stretching mode is unshifted in the deoxy derivative. Thus, in HbI, the frequency of the Fe–His stretching mode does not correlate with the dissociation constant of the last oxygen molecule as found in human hemoglobins. In turn, this finding indicates that the iron–histidine bond does not play the same role in heme–heme communication in HbI as it does in the vertebrate hemoglobins. The low-frequency modes, which are sensitive to the conformation of the peripheral substituents on the heme, differ substantially from those in mammalian hemoglobins. The results show that in the dimeric hemoglobin the interactions between the heme and the protein pocket are substantially different and are consistent with a mechanism of cooperativity involving direct communication between the two hemes.

The cooperative hemoglobin (HbI) from the mollusc *Scapharca inaequivalvis* is unique in many respects. It forms a dimer of identical subunits, in contrast to vertebrate hemoglobins, which are tetramers of two distinguishable subunits. The two heme groups in HbI are in close contact across a novel subunit interface formed by the E and F helices (Royer *et. al.*, 1989). Across this interface, the two heme groups are in direct structural contact via a hydrogen-bonding network. The Fe-Fe distance between the hemes is only 16.7 Å in the deoxy form of the protein, and the hemes are linked through one of their propionates by hydrogen bond bridges formed by Asn residues (Asn^{100}) (Royer *et. al.*, 1989). In the CO-bound form of the protein, the Fe–Fe distance increases to 18.4 Å, and the propionates are bridged by water molecules (Royer *et al.*, 1990). Several other interactions that link one heme group to the F helix of the contralateral subunit differ in the CO-liganded and deoxygenated protein contributing further to direct heme–heme communication. The amino acid residues involved in such interactions, namely, Lys^{96}, Phe^{97}, and Asn^{100}, are all within 1.5 helical turns of the proximal histidine (His^{101}). In addition, Phe^{97} is in contact with the heme and the proximal histidine in the deoxy form of the protein but moves to the subunit interface in the ligand-bound form. These features provide a radically different structural basis for cooperativity in oxygen binding in HbI with respect to that in vertebrate hemoglobins.

The ligand-linked motions of the residues which are near the heme give rise to marked tertiary transitions in the heme environment and to subtle quaternary rearrangements of the subunits (Royer *et al.*, 1989, 1990). For example, one subunit rotates by 3.4° and translates by 0.1 Å with respect to the other in the comparison of the deoxy to the CO-bound forms, in contrast to a corresponding rotation and translation of 15° and 0.8 Å in mammalian hemoglobins. The absence of large quaternary structure changes is reflected in the insensitivity of HbI to allosteric effectors (Chiancone *et al.*, 1981), and represents a further difference in the molecular machinery operative in HbI with respect to vertebrate hemoglobins.

Concerning the heme pocket itself, several spectroscopic markers, e.g., the small hyperfine shift of the $N_\delta H$ proton resonance (Inubushi *et al.*, 1988) and the broad anisotropic EPR *g* signal of the Co–porphyrin-containing protein (Verzili *et al.*, 1982), indicate that in the deoxy derivative the iron–proximal histidine bond is strained. In part, this strain may result from the van der Waals interactions of Phe^{97}, which packs against the heme and the histidine in the deoxy protein (Royer *et al.*, 1990).

The distal side of the pocket is characterized by the presence of a leucine residue (Leu^{73}) in the position corresponding to Val E11 in vertebrate hemoglobins (Royer et al., 1992). The Leu^{73} side chain packs against the heme in the deoxy structure and moves by about 1 Å in the direction of the heme plane in the CO derivative as the heme becomes more planar. In an unliganded subunit, it may therefore hinder access of the ligands to the iron atom.

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In HbI, ligand binding induces only subtle quaternary structural changes as indicated above, and allosteric effectors are unknown. However, HbI reacted with *p*-(chloromercuri)benzoate (PMB) at Cys^{92} (HbI–PMB) has been studied as modification of Cys^{92}, the only cysteine residue in the globin chain and located at the subunit interface, gives rise to subunit rearrangements that are reflected in changes both of the asymptotes of the oxygen binding curve and in the cracking of the carbonmonoxy-HbI crystals. In particular, in HbI–PMB, the oxygen affinity is increased 20-fold in comparison to the native protein, and cooperativity is lost (Boffi *et al.*, 1987).

The present resonance Raman scattering study was undertaken to probe the bonds involving the heme group in deoxy-HbI and ligand-bound HbI and to assess possible differences between vertebrate hemoglobins and the molluscan protein. The spectra of the deoxy, CO-bound, and O_2-bound derivatives were examined and compared to the same derivatives in human adult tetrameric hemoglobin (HbA). Significant differences between HbI and HbA were found in each form of the protein. These differences are consistent with the postulate that the interactions between the heme and its pocket are substantially different in HbI than in HbA. Thus, the data support a novel mechanism of cooperative interactions that was proposed based on the crystallographic analysis of ligand-bound and ligand-free forms of the protein (Chiancone *et al.*, 1990).

MATERIALS AND METHODS

Scapharca inaequivalvis HbI was isolated and purified according to established methods (Chiancone *et al.*, 1981). Human HbA was freshly prepared by the method of Rossi-Fanelli *et al.* (1958). Myoglobin (sperm whale) was obtained from Sigma and filtered before use.

Samples for resonance Raman measurements were prepared in 0.1 M phosphate buffer at pH 7.0 and at a protein concentration of 150–250 μM heme. For the spectra of the deoxy derivative, the sample was deoxygenated by flushing N_2 in the cell, and a small amount of fresh sodium dithionite solution was added just before the measurements. *p*-(Chloromercuri)benzoate (PMB)-modified HbI was prepared as reported by Boffi *et al.* (1987). The Raman measurements were carried out by a resonance Raman difference technique (Rousseau, 1981). Samples were placed in a cylindrical cell with a partition along the diameter such that the samples to be compared are illuminated alternatively by the laser as the cell is rotated. Scattered light was dispersed with a Spex monochromator (Spex Industries, Metuchen, NJ) and detected by a photomultiplier which was interfaced to an AT&T personal computer. All the spectra were measured at room temperature. For ^{13}CO[ICON, Marion, NY; 99% ^{13}C (atom percent)] isotope studies, deoxygenated HbI was placed in each side of a split rotating cell under an N_2 atmosphere, and both compartments were sealed with rubber stoppers. Either ^{13}CO or ^{12}CO was injected directly into the sample. For the $^{18}O_2$ [ICON; 97% ^{18}O (atom percent)] measurements, the degassed sample, sealed in the cell, was flushed several times with the oxygen isotope at a partial pressure of about 1 atm.

Incident laser frequencies of 441.6 and 413.1 nm were used in the resonance Raman measurements. For measurements of the CO derivatives, the laser power was decreased to about 2 mW by an optical filter to minimize photodissociation.

RESULTS

Deoxy Derivatives. The resonance Raman spectrum of deoxy-HbI is compared with those of deoxy-HbA and deoxy-Mb in Figure 1A,B. In the high-frequency region in Figure 1B (1000–1700 cm^{-1}), the spectra of all three of these globins are quite similar. However, in the low-frequency region shown in Figure 1A (150–1000 cm^{-1}), the spectra are very different. The Fe–His stretching mode is located at 203, 216, and 220 cm^{-1} in HbI, HbA, and Mb, respectively. HbI has a line at 287 cm^{-1} which is absent in deoxy-HbA and only a weak shoulder in deoxy-Mb. The region between 330 and 500 cm^{-1} is very different; in particular, the lines at 372, 412, and 494 cm^{-1} are different for each protein. The pair of lines at 758 and 788 cm^{-1} in HbI have the same relative intensity as the corresponding pair in Mb but a different relative intensity than that in HbA.

Carbon Monoxide Derivatives. The spectrum of CO-bound HbI from 100 to 1000 cm^{-1} is compared to those from CO-bound HbA and Mb in Figure 2. Just as for the deoxy case, in the high-frequency region, the spectra from each of the proteins are very similar (data not shown). However, in the low-frequency region, the spectra are different as shown in Figure 2. In contrast to HbA, in HbI a well-defined line occurs at 350 cm^{-1}, and another line with a high-frequency shoulder is present at 418 cm^{-1}. To identify the Fe–CO modes, the spectra of ^{13}CO-bound HbI were compared to ^{12}CO-bound HbI (Figure 3). These isotope experiments demonstrate that the iron–carbon monoxide stretching and the iron–carbon–oxygen bending modes, assigned to the lines at 507 and 578 cm^{-1} in CO-bound HbA, respectively (Yu *et al.*, 1983), are located at 517 [$\Delta(^{12}C-^{13}C) = 7\ cm^{-1}$] and 582 cm^{-1} [$\Delta(^{12}C-^{13}C) = 16\ cm^{-1}$] in HbI and are characterized by different relative intensities ($I_{bend}/I_{stretch}$ is 0.2 for HbI and 0.1 for HbA). Another isotope-sensitive line, in ^{12}CO-bound HbI, occurs at 1945 cm^{-1} (Figure 3) and shifts to 1900 cm^{-1} in the ^{13}CO-bound form. In HbA, the corresponding mode with ^{12}CO is located at 1951 cm^{-1} and has been assigned to the internal carbon–oxygen stretching vibration.

Oxygenated Derivatives. The low-frequency spectrum of O_2-bound HbI is compared to that of O_2-bound HbA in Figure 4. As in the other derivatives, there are no large differences in the high-frequency region but substantial differences in the low-frequency region. Specifically, the line at 376 cm^{-1} is much stronger in the HbA derivative than in the HbI derivative. However, the line at 419 cm^{-1} is stronger in HbA than in HbI. To assign the weak Fe–O_2 stretching mode, $^{18}O_2$-bound HbI was compared to the ^{16}O-bound derivative. Although the region of the Fe–O_2 stretching mode has other overlapping lines, the difference spectrum in Figure 5 clearly identifies the Fe–$^{16}O_2$ stretch mode at 570 cm^{-1}, very close to the same frequency as in HbA (Brunner, 1974).

PMB-Modified Derivative. The deoxy and the CO-bound derivatives of PMB-modified HbI were compared to the corresponding unmodified derivatives. No significant changes in the CO-bound derivatives were found, and the modes involving Fe–C–O motions were identical (data not shown). For the deoxy derivative, some changes in the low-frequency region were detected as shown in Figure 6, but not in the high-frequency region (data not shown). No change in the peak frequency of the Fe–His stretching mode was found although changes in high-frequency shoulders from 210 to 250 cm^{-1} were observed. The line at 333 cm^{-1} is weaker in the PMB-modified protein than in the unmodified form, and the line at 374 cm^{-1} broadens and shifts to lower frequency.

DISCUSSION

Several normal-mode analyses have been reported for metalloporphyrins and heme proteins [for example, see Abe

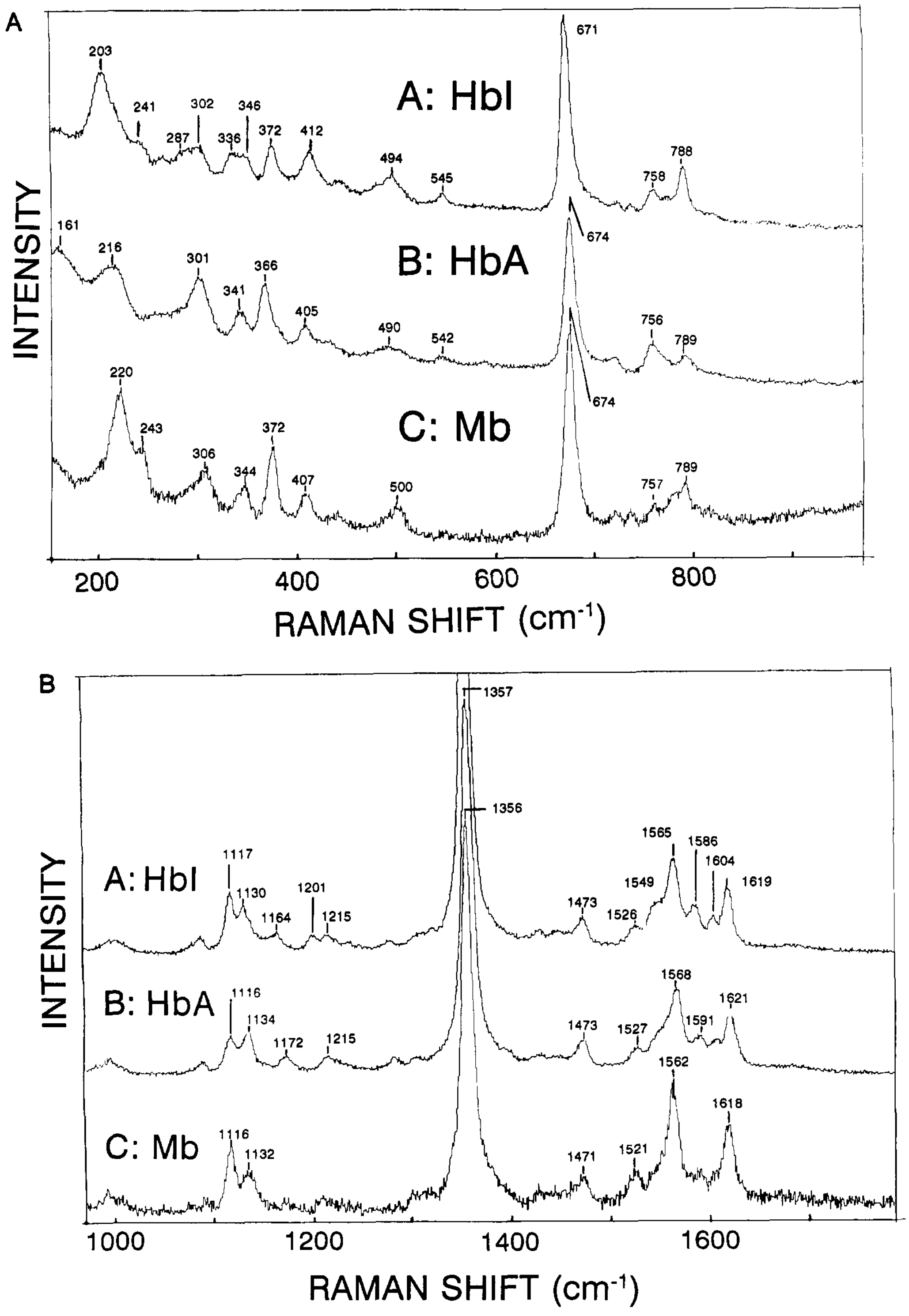

FIGURE 1: Comparison of the resonance Raman spectra of the ligand-free deoxy forms of *Scapharca inaequivalvis* hemoglobin (A: HbI), human adult hemoglobin (B: HbA), and sperm whale myoglobin (C: Mb). Panel A shows the low-frequency region, and panel B shows the high-frequency region. The incident laser wavelength was 441.6 nm, and the power at the sample was about 20 mW.

et al. (1978); Kitagawa and Ozaki (1987), Spiro and Li (1988), and Li *et al.* (1989, 1990a,b)]. Unequivocal assignments have been made for many modes including porphyrin macrocycle modes in the high-frequency region, some of which are sensitive to the π-electron density whereas others are sensitive to the size of the porphyrin core defined by the four nitrogen atoms of the pyrrole rings (Spiro & Strekas, 1984; Choi *et al.*, 1982). In the low-frequency region, the Fe–His stretching mode (Kitagawa *et al.*, 1979; Argade *et al.*, 1984) in the 200–230-cm^{-1} range in the deoxy derivative is of special interest since in vertebrate hemoglobins it is the most sensitive mode to quaternary structure changes (Kitagawa, 1988), although other modes in both the low- and the high-frequency regions also show quaternary structure dependence (Rousseau & Ondrias, 1983). Many of the low-frequency modes are sensitive to heme–protein interactions since they have contributions from peripheral substituents which are conformationally flexible and in van der Waals contact with amino acid residues that comprise the heme pocket. In each of the derivatives of HbI reported on here, the high-frequency region of the Raman spectrum is similar in HbI, HbA, and Mb, demonstrating that in these globins the electron density within

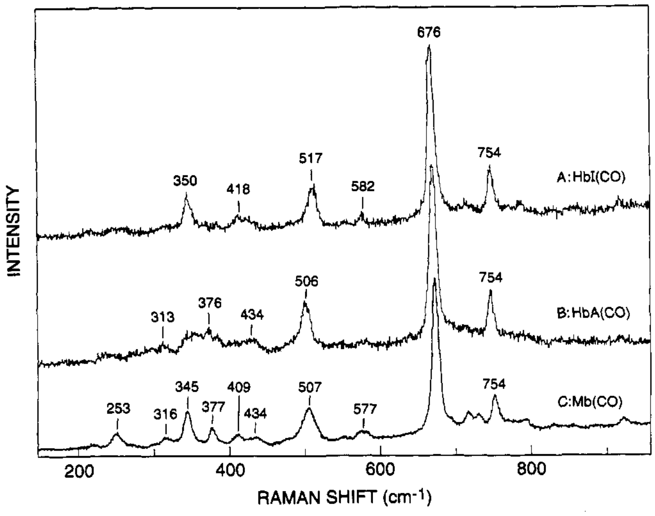

FIGURE 2: Comparison of the low-frequency resonance Raman spectra of the carbonmonoxy derivatives of *Scapharca inaequivalvis* hemoglobin (A: HbI), human adult hemoglobin (B: HbA), and sperm whale myoglobin (C: Mb). The laser excitation wavelength was 413.1 nm, and the power at the sample was about 2 mW.

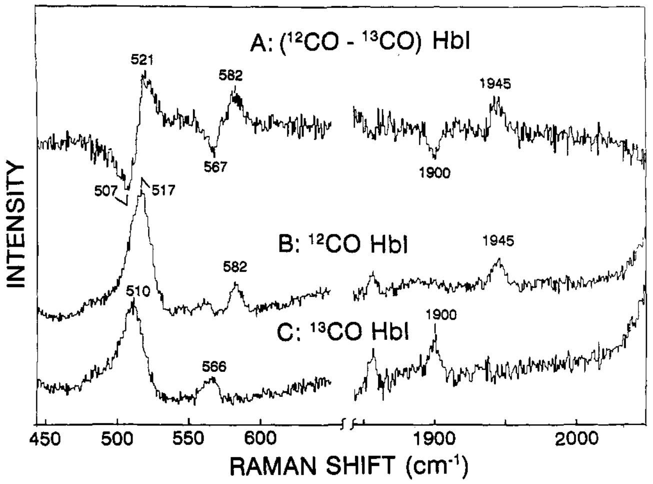

FIGURE 3: Carbon isotope dependence of the resonance Raman spectra of (CO)HbI. Only the modes at 517, 582, and 1945 cm^{-1} in ^{12}COHbI (spectrum B) show any ^{13}C (spectrum C) isotope shifts. From the shifts evident in the individual spectra and the difference spectrum (A), the modes are assigned as the Fe–CO stretch, the Fe–C–O bend, and the C–O stretch, respectively. The laser conditions were the same as those in Figure 2.

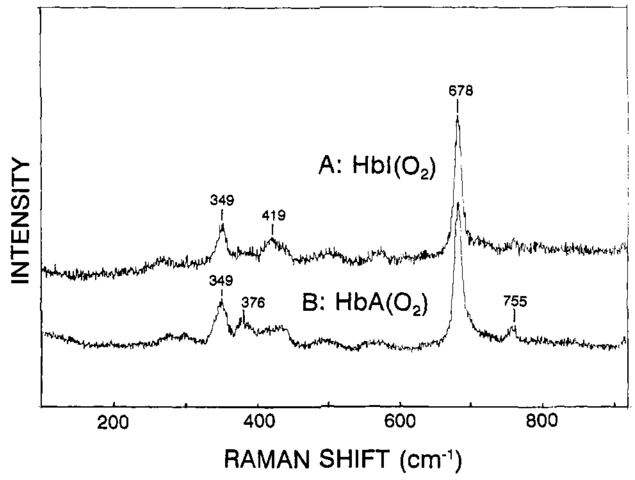

FIGURE 4: Comparison of the low-frequency resonance Raman spectra of the oxygenated hemoglobins of *Scapharca inaequivalvis* (A: HbI) and human adult hemoglobin (B: HbA). The laser excitation wavelength was 413.1 nm, and the power at the sample was about 10 mW.

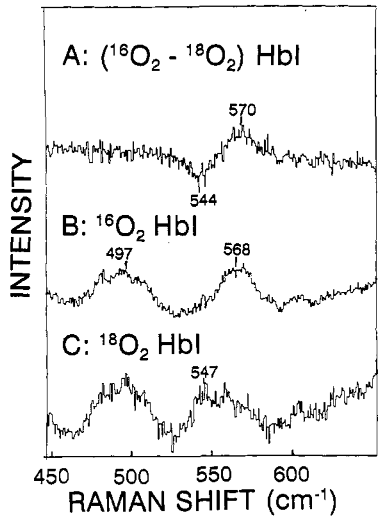

FIGURE 5: Oxygen isotope dependence of the resonance Raman spectra of HbI(O_2). The only line displaying an isotope shift in comparison of the $^{18}O_2$ derivative (spectrum C) and the $^{16}O_2$ derivative (spectrum B) is that at 568 cm^{-1} in HbI($^{16}O_2$). It is assigned at the Fe–O_2 stretching mode. The difference spectrum (A) illustrates that the line at 497 cm^{-1} has no isotope dependence. The laser conditions were the same as those in Figure 4.

the porphyrin macrocycle and the basic porphyrin structure are not sensitive to the protein structural differences. However, the low-frequency region of the spectrum displays substantial differences, indicating that the interactions between the heme and the amino acid residues of the pocket are dissimilar.

Deoxygenated Derivative. In deoxygenated derivatives of hemoglobins, the Fe–His stretching mode has been very well characterized since it displays large quaternary structure dependent changes. Studies of this mode from a variety of vertebrate hemoglobins have confirmed conclusions based on crystallographic studies that the iron–histidine bond is the central conduit linking the protein structure to the ligand binding site (Friedman *et al.*, 1982; Friedman, 1985; Rousseau & Friedman, 1988; Kitagawa, 1988). In HbI, at variance with HbA, the proximal histidine is not the sole residue that links the heme group to the apoprotein since each heme establishes several interactions across the subunit interface with amino acid residues belonging to the F helix of the other subunit (Royer *et al.*, 1989, 1990). The relevance of such interactions in heme–heme communication has been brought out recently by stopped-flow experiments designed to monitor the cleavage of the proximal Fe–N$_\epsilon$ bond at low pH through the rate of carbon monoxide binding. In HbI, cleavage of the Fe–His proximal bond is cooperative, indicating that this bond does not represent the only means to transmit information about the ligand binding state of the heme through the protein structure (Coletta *et al.*, 1990).

In deoxy-HbI, the Fe–His stretching frequency occurs at 203 cm^{-1}, by far the lowest value observed to date for hemoglobins and myoglobins, comparable only to the frequency assigned to the α-subunits in tetrameric human hemoglobins (Ondrias *et al.*, 1982; Rousseau & Friedman, 1988). It is of interest that the hyperfine shift of the N$_\delta$H group (Inubushi *et al.*, 1988) and the broad *g* signal of Co-substituted HbI (Verzili *et al.*, 1982) also indicated a similarity of the structural and electronic features of the deoxy-HbI heme and the α-subunits of the human hemoglobin tetramer. The low frequency of the Fe–His stretching mode in deoxy-HbI, like the NMR and EPR data just recalled, points to a reduced strength for the iron–nitrogen bond. The X-ray crystal

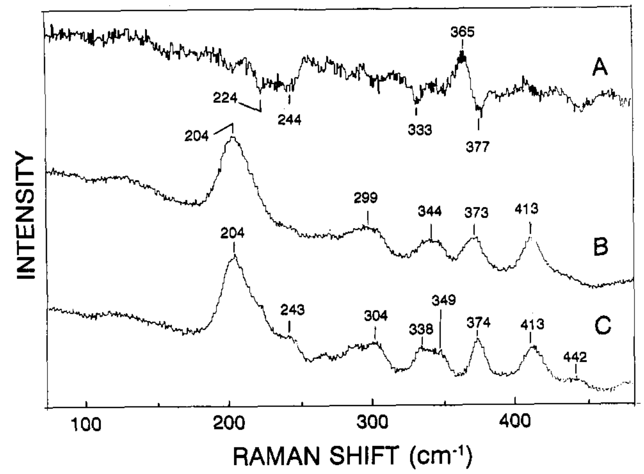

Figure 6: Comparison of the low-frequency region of the resonance Raman spectra of unmodified deoxy-HbI (spectrum C) and PMB-modified deoxy-HbI (spectrum B). The difference spectrum A [=(B) – (C)] brings out the spectral changes. The incident laser wavelength was 441.6 nm, and the power at the sample was about 20 mW.

structure of deoxy-HbI (Royer *et al.*, 1990) shows that the aromatic ring of Phe^{97} packs against the proximal histidine and could thereby exert a steric strain, weakening the iron–nitrogen bond. This strain would be released in the ligand-bound derivative as Phe^{97} swings out of the heme pocket into the interface during the conformational transition that accompanies ligand binding (Royer *et al.*, 1989). Since the Fe–His mode has not been assigned in the ligand-bound derivatives, the effect of the movement of Phe^{97} on the Fe–His bond cannot be assessed directly. In addition to steric strain, a very weak hydrogen bond at N_δ in the histidine as suggested by crystallographic and NMR data could also contribute to the low frequency.

The Fe–His stretching mode in the resonance Raman spectrum of HbI displays a further difference relative to that of human hemoglobin. It is more intense and symmetric (Figure 1A). Both characteristics have been correlated with the orientation (azimuthal angle) of the imidazole plane of the histidine with respect to the pyrrole N_1–N_3 axis (Bangcharoenpaurpong *et al.*, 1984). On the basis of considerations of the azimuthal angle, the maximum overlap between the σ^*-orbital of the Fe–His bond and the π^*-orbital of the porphyrin macrocycle would be achieved for the eclipsed orientation and thus give rise to the highest Raman scattering intensity. The crystallographic structure of deoxy-HbI and CO-bound HbI and NMR data on the cyano-met derivative (McGourty *et al.*, 1989) indicated that the orientation of the imidazole plane of the histidine is *not* eclipsed but instead lies between the pyrrole N_1–N_3 axis and the pyrrole N_2–N_4 axis. In the CO-bound form, the imidazole plane lies nearly exactly between these two axes, but in the deoxy form, it appears to be pushed away from that position somewhat by the presence of the phenylalanine in the pocket. Thus, the present results in which the Fe–His stretching mode is very strong and the imidazole orientation is far from eclipsed indicate that the hypothesis of Bancharoenpurpong *et al.* (1984) does not hold in HbI.

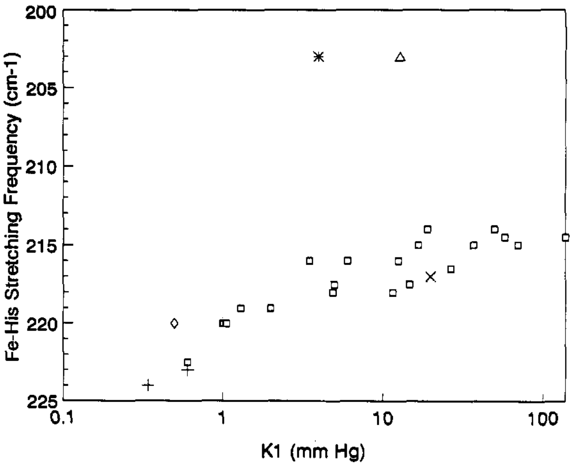

Figure 7: Correlation, reported by Matsukawa *et al.* (1985), of the constant for dissociation of the last oxygen molecule from various mutants of HbA and the frequency of the iron–histidine stretching mode. The squares represent values of a variety of tetrameric hemoglobins obtained under various conditions. The point designated by the triangle is for HbI, by the diamond is for myoglobin, by the star is for the $\alpha_2\beta_2$(CN) hybrid, and by the x is for the α_2(CN)β_2 hybrid, and those points designated by the crosses are for the isolated α and β chains.

In mutants of human hemoglobin, there is a correlation between the frequency of the Fe–His stretching mode and K_1, the constant for dissociation of the last oxygen molecule from oxy-Hb (Matsukawa *et al.*, 1985), as may be seen in Figure 7. This correlation is also valid from the isolated α and β chains, for myoglobin, and for the β-subunits in the Hb tetramer. Only HbI ($\nu_{\text{Fe-His}}$ = 203 cm^{-1}; K_1 = 12.3 mmHg) and the α-subunits in the Hb tetramer [$\nu_{\text{Fe-His}}$ = 203 cm^{-1}; K_1 = 4 mmHg in the $\alpha_2\beta_2$(CN) hybrids] (Nagai, 1977) lie far from this correlation curve, in line with the structural and electronic similarity of these hemes. These data demonstrate that the established relationship between the Fe–His stretching mode frequency and the oxygen affinity which applies to the tetrameric hemoglobins does not hold in HbI. It should be noted that myoglobin falls on the hemoglobin correlation curve even though it is monomeric and thereby noncooperative. Similarly, the isolated subunits also fall in the curve.

In vertebrate hemoglobins, the Fe–His stretching mode has a lower frequency in the T than in the R structure and thus allows the study of the variation in heme–protein interactions as a function of quaternary structure (Rousseau & Ondrias, 1983). The mechanism for cooperativity in HbI based on the X-ray crystal structures of the deoxy and CO derivatives does not entail significant quaternary changes. Moreover, no allosteric effectors are known. However, a quaternary rearrangement of the subunits, significant enough to break the CO-ligated crystals, can be induced by PMB and results in a dramatic increase in oxygen affinity and in the loss of cooperativity. In turn, the oxygen binding curves also point to the occurrence of major structural changes in deoxygenated HbI–PMB as both asymptotes are altered. Yet no changes in the frequency of the Fe–His stretching mode was observed in HbI–PMB with respect to the native protein. This finding indicates that the increase in oxygen affinity does not originate from a modification of the geometric features of the iron–histidine bond, in agreement with kinetic pH titration experiments which showed that PMB modification did not change the p*K* value of the proximal histidine ionization (Coletta *et al.*, 1990).

The data reported here confirm that the iron–histidine bond does not play the same critical role in HbI as it does in vertebrate hemoglobins. Analysis of crystallographic data led to the postulate that in HbI the mechanism of cooperativity involves direct heme–heme interactions resulting from the proximity of the heme groups (Chiancone *et al.*, 1990). The different spectra in the low-frequency region in HbI with respect to HbA are consistent with this postulate since the spectra indicate that the interactions between the peripheral substituents on the heme and the heme pocket are different. These low-frequency modes have complex potential energy distributions involving in-plane and out-of-plane motions of the atoms in the macrocycle as well as motions of the peripheral substituents. Several normal coordinate studies have clarified the assignments (Abe *et al.*, 1978; Kitagawa & Ozaki, 1987; Spiro & Li, 1988; Li *et al.*, 1989, 1990a,b). In the region near 300 cm^{-1}, two modes are assigned—one involving the out-of-plane motion of the methine carbon atoms and the other a bending mode of the vinyl substituents. The lower intensity of the line at 302 cm^{-1} in HbI, with respect to HbA, may reflect a different disposition of the vinyl groups. In the 320–350-cm^{-1} region, HbI has two modes at 336 and 346 cm^{-1}, whereas HbA and Mb have only single modes at 341 and 344 cm^{-1}, respectively. In HbA and Mb, these lines were assigned as ν_8, a mode involving in-plane substituent group bending motion. The splitting of this line in HbI is a further indication of different peripheral group interactions in these two hemoglobins. The mode at 372 cm^{-1} has the same frequency as the corresponding mode in myoglobin but a different frequency than that in HbA (366 cm^{-1}). This is interesting since this line, an out-of-plane bending mode of the peripheral substituents, has been assigned as being the most sensitive to isotopic substitution of atoms of the propionate groups (S. C. Hu and T. G. Spiro, unpublished results), suggesting that the environments for the propionates are the same in HbI as in Mb, but different from HbA. From the structures of these proteins, the origin of these differences is not clear. In all cases, the heme lies in a nonpolar environment, but the propionates have polar contacts. Changes in the mode at 372 cm^{-1} in transient resonance Raman spectra of HbI (Rousseau *et al.*, 1993b) are consistent with changes in the environment of the propionates upon ligand binding. The mode at 412 cm^{-1} is assigned as another in-plane vinyl bending mode, and its frequency difference with respect to HbA and Mb is a further indication of the unique environment of the heme in HbI. The largest difference in the comparison of the PMB-modified protein with the native form occurs in the line at 374 cm^{-1}. Thus, its assignment, as being most sensitive to the propionate groups, is evidence that these groups change in the PMB-modified protein in contrast to the absence of any changes in the frequency of the Fe–His stretching mode.

Ligand-Bound Derivatives. An analysis of the low-frequency region of the CO-bound (Figure 2) and O_2-bound (Figure 4) proteins leads to the same conclusions as may be drawn from the deoxy derivative: namely, the spectral differences result from changes in the lines associated with the peripheral substituents. In the CO-derivatives, ν_8, the substituent bending mode at 350 cm^{-1}, is very different for HbI(CO), HbA(CO), and Mb(CO). The mode assigned as having a large contribution from the propionates at about 376 cm^{-1} is absent in the spectrum of HbI(CO), and the vinyl group sensitive mode at 418 cm^{-1} is also different in the spectra of all three globins. Just as in the CO derivatives, in the O_2 derivative the mode at 376 cm^{-1} is absent in HbI, and the vinyl region is very different.

The resonance Raman spectrum of carbonmonoxy-HbI also provides information on the characteristics of the distal site. Comparison with the HbA spectrum brings out important differences in the geometry of the CO-bound molecule. The frequencies and intensities of the Fe–CO stretching, Fe–C–O bending, and C–O stretching modes (Figure 3) indicate that the CO molecule is tilted from the normal to the heme plane. This is apparent from the relative intensities of the δ(Fe–C–O) bending to the ν(Fe–CO) stretching peaks. Analysis of a series of model compounds in which steric hindrance causes the Fe–C–O moiety to be tilted away from the normal to the porphyrin plane has revealed that the intensity of the bending mode (I_{bend}) relative to the stretching mode ($I_{stretch}$) increases with tilt angle (Yu *et al.*, 1983). For Hb, $I_{bend}/I_{stretch} = 0.1$, in contrast to our finding (Figure 3) that in HbI the ratio is ~0.2. Hence, in HbI, the geometry of the Fe–C–O grouping is more "myoglobin like" ($I_{bend}/I_{stretch} = 0.25$). From the frequencies of the three modes involving CO [ν(Fe–CO) = 517 cm^{-1}, ν(Fe–C–O) = 582 cm^{-1}, and ν(CO–O) = 1945 cm^{-1}], it has been possible to estimate an Fe–C–O bond angle of 171 ± 5°, in the limit of the three-body oscillator approximation (Yu *et al.*, 1983). Interestingly, in HbI–PMB, the frequency values and intensities relative to the above-mentioned peaks are unchanged, indicating that PMB modification is not reflected in an alternation of the geometry of the distal site.

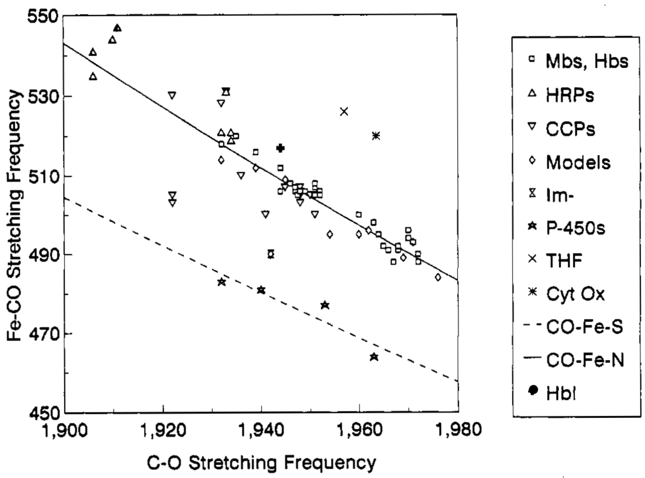

FIGURE 8: Correlation curves showing the relationship between the frequency of the Fe–CO stretching mode and that of the C–O stretching mode. The solid line is the fit for those proteins and porphyrins with nitrogenous axial ligands trans to the bound CO, and the dashed line shows the correlation of proteins with a cysteinal proximal ligand. The x designates the point for a porphyrin with THF (tetrahydrofuran) as its proximal ligand, and the star is the point resulting from cytochrome *c* oxidase. The darkened cross corresponds to HbI. It lies on the nitrogenous ligand correlation curve.

It is well-known that when CO and histidine (or imidazole) are the axial ligands in a porphyrin system, there is a correlation of the Fe–CO and C–O stretching frequencies (Yu *et al.*, 1983; Tsubaki *et al.*, 1986; Uno *et al.*, 1987; Smulevich *et al.*, 1988; Kerr & Yu, 1988; Morikis *et al.*, 1989; Spiro *et al.*, 1990; Nagai *et al.*, 1991; Rousseau *et al.*, 1993a). Such a correlation is presented in Figure 8 where the filled cross is the point originating from HbI. It lies well within the uncertainties of the data and thereby demonstrates that even though the Fe–CO frequency is very high compared to most other hemoglobins and myoglobins, the electronic structure is not anomalous, unlike cytochrome *c* oxidase, for example, which does not lie on the curve.

Measurements on the oxygenated derivative allowed determination of the frequency of the Fe–O_2 stretching mode (Figure 5). Its frequency value is essentially the same as that observed for HbA(O_2) (Brunner, 1974; Nagai *et al.*, 1980), Mb(O_2) (Van Wart & Zimmer, 1985), and cytochrome *c* oxidase–O_2 (Han *et al.*, 1990a,b). In general, the frequency of the Fe–O_2 stretching mode does not appear to be very sensitive to proximal and distal interactions. Thus, its frequency would be expected to be similar to these other heme proteins.

CONCLUSION

The data reported here present the first comprehensive study of the Raman spectra of *Scapharca inaequivalvis* HbI. For the deoxy, CO-bound, and O_2-bound derivatives, it was found that the low-frequency region of the resonance Raman spectra of HbI differs from that of HbA although the spectra in the high-frequency region are quite similar. Since the modes in the low-frequency region of the spectrum have contributions from peripheral substituents, the data demonstrate that the interactions between the heme and the amino acid residues in the heme pocket of HbI are not the same as those in HbA. Also, the frequency and intensity of this iron–histidine stretching mode are not the same as those in hemoglobins and myoglobins; it does not correlate with the oxygen affinity, and it is insensitive to PMB modification which increases oxygen affinity and abolishes cooperativity.

Taken together, the results reported here confirm the proposed mechanism of cooperativity (Chiancone *et al.*, 1990) in HbI which is fundamentally different from that in vertebrate hemoglobins. Instead of heme–heme communication occurring by a conduit orginating along the iron–histidine axis as in vertebrate hemoglobin, cooperativity arises from interactions involving the peripheral substituents on the heme groups. Owing to the proximity of the hemes in the dimer, changes near one heme may be directly communicated to the other. In vertebrate tetrameric hemoglobins in which the heme–heme distance is much longer, heme–heme communication must rely on the change in the position of the proximal histidine with respect to the heme. Thus, the mechanism of cooperativity depends on how the hemes are positioned with respect to each other by the protein matrix.

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