Unusual Rocking Freedom of the Heme in the Hydrogen Sulfide-Binding Hemoglobin from *Lucina pectinata*

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Abstract: Hemoglobin I (HbI) from the clam *Lucina pectinata* is, in its natural environment, a hydrogen sulfide (H_2S)-transport heme protein. The resonance Raman (RR) spectrum of the metaquo and deoxyHbI species shows a very weak intensity peak at 370 cm^{-1} that corresponds to the normal mode of the heme propionates. This suggests the presence of a moderate hydrogen bonding between Arg99 and the heme-7-propionate. However, the RR spectra of the metcyano, carbonmonoxy, and oxy HbI derivatives reveal the absence of the propionate vibrational frequency at 370 cm^{-1}. The mode is insensitive to the oxidation state of the heme iron, but disappears when the HbI−ligand moiety is formed. These results propose the existence of flexible propionate groups which can result in a weaker hydrogen bond upon heme ligand binding. The longitudinal relaxation time (T_1) ^1H NMR data for the paramagnetic metcyano complex of HbI suggested that the 17.90 ppm signal belongs to the heme-6-propionate α′ protons (6-$H_{\alpha'}$). In relation to other myoglobins, the large difference in chemical shifts of this signal is attributed both to the lack of hydrogen bonds between the heme-6-propionate group and amino acid residues and to a flexible orientation of the side chain with respect to the heme plane. The data predict a model where the heme group of HbI is tightly bound to His96 (ν_{Fe-His} at 218 cm^{-1}), but due to the absence of strong hydrogen bonding interactions between the heme propionates and the nearby amino acids, the heme is not firmly anchored. Thus, relative to other heme proteins, the heme group of HbI from *Lucina pectinata* presents a rocking freedom that facilitates the binding between the heme and the in-coming ligand.

Introduction

The clam *Lucina pectinata*, which is found in the sulfide-rich tropical coasts of Puerto Rico, contains three hemoglobins HbI, HbII, and HbIII. Each hemoglobin has different physical-chemical properties.[1,2] Hemoglobin I (HbI) is a sulfide-reactive heme protein in its natural environment. The other two hemoglobins, HbII and HbIII, do not bind hydrogen sulfide, they are used as oxygen-transport proteins. The ability of HbI to reversibly bind hydrogen sulfide with high affinity is unusual for a hemoglobin.[1,2] In human hemoglobin, histidine E7 helps to control oxygen affinity through hydrogen bonds with the ligand. This amino acid is a conserved residue in many other hemoglobins and myoglobins. However, X-ray crystallographic analysis of the metaquo form of HbI from *L. Pectinata*, at 1.5 Å resolution, showed that the distal E7 residue is a glutamine amino acid instead of the usual histidine.[3] Other notable substitutions in the HbI heme pocket include Phe29(B10), Leu46(CD3), and Phe68(E11). The aromatic environment present in the near HbI chromophore stands as the molecular basis for the very high affinity of *Lucina pectinata* for hydrogen sulfide as proposed by Rizzi and collaborators.[3,4] This peculiar arrangement may play a significant role for the stabilization of heme-bound hydrogen sulfide through aromatic-electrostatic interactions. We reported an infrared spectroscopy study of HbI from *L. pectinata* and of a His64(E7) → Gln, Leu29(B10) → Phe, Val68(E11) → Phe triple mutant from sperm-whale myoglobin (SW Mb) carbonmonoxide derivatives.[5] Both proteins showed a larger population of the A_3 conformer at 1936 cm^{-1} than the A_0 or $A_{1,2}$ conformers when compared to wild-type SW Mb. The preference of the A_3 conformer in these proteins is produced by the existence of the aromatic phenylalanine residues next to the heme group. The triple mutant having both the distal Gln substitution at position E7 and the phenylalanine substitutions at position B10 and E11 is, of all Mb mutants compared, the one with the most similar CO association rate constant to the A_3 conformer of HbI. The synergetic effect of the three unique residues in HbI (Gln64, Phe29, Phe68) was proposed to control the population of the conformational states and the CO association rate of this protein.[5] Recent X-ray studies[6] of the aquo derivatives triple mutant SW Mb (His64(E7) → Gln, Leu29(B10) → Phe, Val68-(E11) → Phe) showed an increase of ∼700-fold in the sulfide affinity of SW Mb. On this regard, a difference in the orientation of the Phe68(E11) ring may account for the difference in affinity between *Lucina* HbI and the SW Mb triple mutant. Comparison of the X-ray crystallographic structure of the metaquoHbI complex with the NMR solution structure of

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metcyanoHbI derivative suggested that Gln67(E7) may serve as either a H-bonding acceptor or donor to bound ligands.[6]

Furthermore, new studies[7] indicated a unique orientation of the heme 2-vinyl group relative to other heme proteins. The RR spectra of the HbICO, metHbICN, metHbIH_2O, HbIO_2, and deoxyHbI heme derivatives showed a band at 1621 cm^{-1} and a shoulder at 1626 cm^{-1}, indicative of an out-of-plane position for one of the vinyls relative to the other one. Spin−lattice relaxation properties of protons in the metHbICN complex also suggested a unique orientation for the heme 2-vinyl group of HbI. The longitudinal relaxation times (T_1) for the 2-H_α, 2-$H_{\beta c}$, and $H_{\beta t}$ protons is 120, 115, and 135 ms, respectively. The data from both techniques suggested an out-of-plane and *trans*-oriented 2-vinyl group, and an in-plane and *cis*-oriented 4-vinyl group for the low spin complexes of HbI. These results imply that the electron-withdrawing character of the out-of-plane vinyl group contributes to the stability of the heme Fe^{3+} oxidation state, facilitates the binding of the H_2S ligand, and promotes the stability of this ferric H_2S complex.

These previous works illustrate how in HbI the distal site environment and heme peripheral groups influence the heme ligand interactions. However, it is not clear if there is any type of interaction between other heme peripheral substituents and nearby amino acids that can be related to the peculiar characteristics of this hydrogen sulfide-binding protein. In the majority of the heme-proteins, the heme binding to the apoprotein is in part stabilized through a hydrogen bond network between the heme 6- and 7-propionate groups and the polar amino acids in the polypeptide chain that surround the heme chromophore.[8a,b,9a,b,10−13] These electrostatic interactions contribute to the stability of the folded haloprotein. The charged propionate groups also can contribute to modulate the oxidation reduction potential of the heme iron.[12,14] To elucidate the contribution of the heme peripheral propionate groups to *Lucina pectinata's* HbI properties, resonance Raman (RR) and proton nuclear magnetic resonance (^{1}H NMR) studies on metcyano, carbonmonoxy, oxy, deoxy, and metaquoHbI derivatives were conducted. Both RR and ^{1}H NMR data are consistent, with the available X-ray data[3] on the presence of moderate hydrogen bonding between the heme's propionates and its environment. On this regard, the results suggest a model in which the heme's propionate groups in HbI are not tightly anchor to the polypeptide chain due to the absence of a strong hydrogen bond networking. This behavior can be correlated with changes on the 370 cm^{-1} propionate vibrational mode upon ligand binding.

Experimental Section

Sample Preparation. Isolation of hemoglobin I from *Lucina pectinata* and preparation its heme-ligand complexes was done according to the literature.[1,2,5] To prepare the metaquo complex, the oxy HbI was oxidized with 10% excess potassium ferricyanide to obtain ferric HbI. To eliminate the potassium ferricyanide excess, the ferric HbI solution was passed through a column of Shephadex G-25 superfine (Pharmacia), developed with 100 mM potassium phosphate buffer at pH 7.5. MetcyanoHbI complex was prepared by adding 20 mM NaCN to the metaquo solution. OxyHbI derivative was used as obtained from the purification procedures. The deoxy derivative was prepared by adding a slight excess of sodium dithionite to the oxyHbI solution. For the HbI carbonmonoxy complex, the heme protein was reduced by using a few grains of sodium dithionite in phosphate buffer at pH 7.5, in an oxygen free atmosphere. The sample was stirred under a carbonmonoxide atmosphere for several minutes. The sodium dithionite excess was removed by changing the solvent to carbonmonoxide-saturated 0.1 M potassium phosphate buffer, pH 7.5, in an Amicon ultrafiltration cell with Y-10 membranes. For the NMR experiment, the metcyanoHbI complex was prepared by dissolution of lyophilized ferric HbI in a 200 mM NaCl/20 mM NaCN solution in 100% D_2O, to obtain a ∼4 mM solution. The pH was adjusted to 7.8 with DCl or NaOD. The characteristic Soret bands for these complexes,[1,2] at 407 nm for metaquoHbI, 421 nm for metcyanoHbI, 416 nm for oxyHbI, 431 nm for deoxyHbI, and 420 nm for carbonmonoxyHbI, were used to monitor the formation of the complexes.

Resonance Raman Measurements. Resonance Raman (RR) scattering was obtained for metcyano, carbonmonoxy, oxy, deoxy, and metaquoHbI heme-ligand moieties at room temperature. The hemoglobin concentration in each preparation was approximately 100 μM. Excitation at 413.1 nm with a Kr^+ ion laser (Coherent Innova 400) was used to obtain the RR spectrum of the metcyano, oxy, metaquo, and carbonmonoxyHbI complexes, while the deoxyHbI complex RR spectrum was excited with a 437.0 nm dye laser, pumped with an Ar^+ ion laser (Coherent Innova 400). A back illuminated CCD detector coupled to a modified Spex 1401 single monochromator and Notch filters were used to record the RR spectra. The monochromator calibration was verified before and after the Raman scattering with carbon tetrachloride. All HbI solutions were spun in a spinning cell during the acquisition of the RR spectra to minimize photoinduced damage to the samples. Laser power was near 10 mW at the sample and each spectrum was averaged for 15 min. A visible spectrum (from 400 nm to 700 nm) was obtained before and after the RR measurement to verify sample integrity.

^{1}H NMR Measurements. ^{1}H NMR spectra were collected from both GE Ω 600 MHz and Varian 300 MHz spectrometers. 1-D ^{1}H NMR spectra from the GE Ω 600 MHz spectrometer were collected by using 2048 scans, 15K data points, and a spectral width of 26315 Hz. NOESY spectra were collected at 19.7 °C over a spectral window of 22222 Hz, using 2048 complex points, a block size of 2048 was used, and a total of 512 blocks were collected. The mixing time used for NOESY was 100 ms. Longitudinal relaxation time (T_1) was obtained from inversion−recovery experiment. The data were generated with use of the Varian spectrometer operating at 300 MHz, applying the standard inversion−recovery sequence (t_d−π−τ−π/2−Aq), the relaxation delay (t_d), and the acquisition time (Aq) being 750 ms and 69 ms, respectively. The spectral width used for the 300 MHz measurements was 13000 Hz. The T_1 experiments were performed at 30 °C and pH 8.6. Chemical shifts and T_1 were compared with results for elephant and sperm whale myoglobin reported earlier.[15] The T_1 was obtained with the null method ($T_1 = t_{null}/\ln 2$). 1-D spectra for the Curie plot were carried out from 8 to 50 °C in the 300 MHz spectrometer, using the same spectral window as described above. The NMR data were processed with the Felix 230 and the NMR TRIAD program. A 30°-shifted sinebell apodization process was used prior to zerofilling to 2048 × 2048 data points and Fourier transformation. Chemical shifts were referred to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Results

Figure 1 compares the low-frequency RR spectra of metaquo horse heart myoglobin (HH Mb) with metaquoHbI and deoxy-

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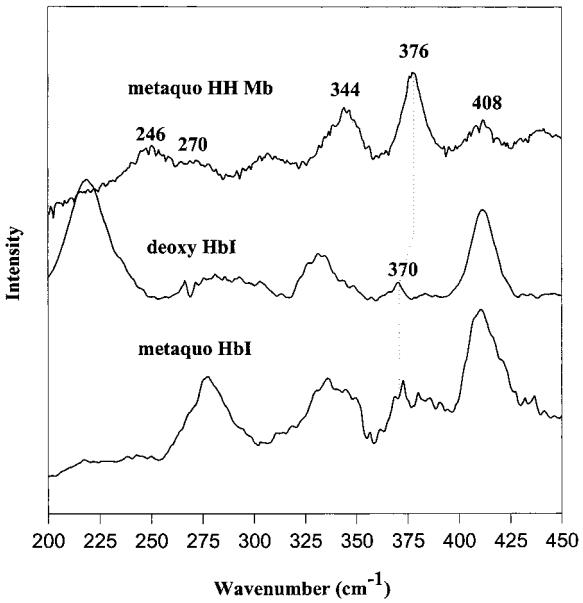

Figure 1. Resonance Raman spectra of metaquo Mb(HH), deoxyHbI, and metaquoHbI. The low-frequency region of deoxyHbI and metaquoHbI in comparison with metaquo Mb(HH) shows the small intensity of the propionate band at 370 cm^{-1}.

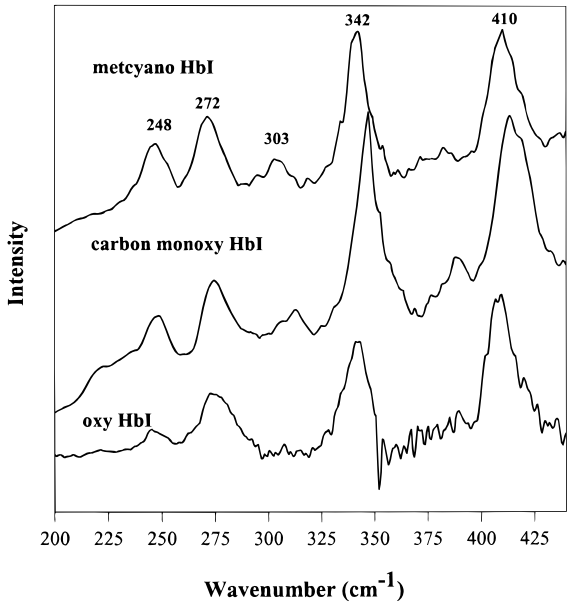

Figure 2. Resonance Raman spectra of metcyano, carbonmonoxy, and oxyHbI. The low-frequency region shows the absence of the propionate band usually found between 366 and 375 cm^{-1}.

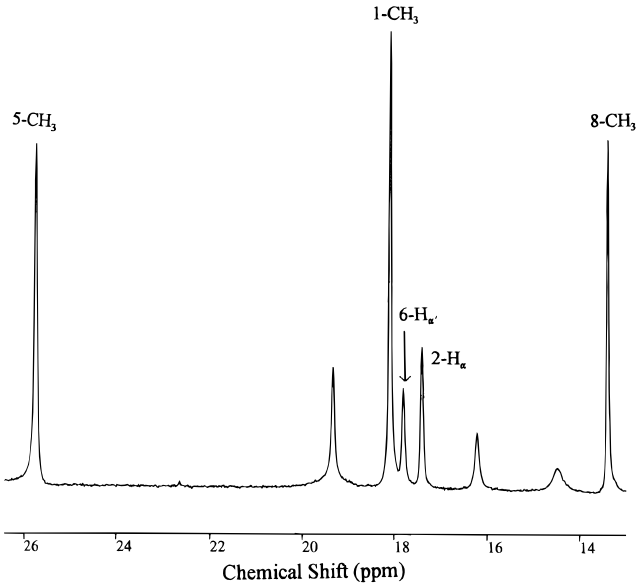

Figure 3. Portion of the downfield hyperfine shifted region of the 600 MHz ^1H NMR spectrum of 4 mM metHbICN paramagnetic complex in H_2O at 19.7 °C, pH 7.8, showing the heme-6-propionate α′ proton resonance line (6-$H_{\alpha'}$). The methyl group signals (1-CH_3, 5-CH_3, and 8-CH_3) were assigned by Silfa and co-workers.[7]

HbI derivatives. Normal mode assignments for the metaquo HH Mb and HbI species were based on the presence or absence of a particular frequency on the RR spectra for the HbI-ligand complexes and upon comparison of these values with those reported in the literature.[16−18] The Fe−N(His) stretch has been observed in the RR spectra of hemoglobins and myoglobins in the 217−224 cm^{-1} region. This mode has been reported for almost all types of heme proteins active only in the deoxy spectrum. A possible explanation for this behavior is that doming of the heme in the deoxy state causes this out-of-plane mode to become active. For the deoxyHbI spectrum this low-frequency region is characterized by a strong band at 218 cm^{-1} assigned to the νFe−N(His) stretching mode. When the metaquo HH Mb and metaquoHbI spectra are compared, they reveal differences in the 250−280, 325−350, and 365−380 cm^{-1} regions. In HH Mb the band at 246 cm^{-1} was assigned to the out-of-plane pyrrole tilting mode. The 270 cm^{-1} band was assigned to the skeletal mode ν_9. The band at 344 cm^{-1} was assigned to ν_8, to a metal pyrrole stretch and substituent bend.[18] The vibrational mode at 376 cm^{-1} corresponds to the $C_\beta-C_c-C_d$ bending motion of the propionates peripheral heme substituents.[16] A significant difference between the HbI and HH Mb spectra presented here is that for the former the propionate band appears with very low intensity at 370 cm^{-1} while for the latter this is a very intense band present at 376 cm^{-1}. Likewise, the deoxyHbI derivative shows a normal Fe−His stretching mode at 218 cm^{-1}, but presents a very weak intensity vibration at 370 cm^{-1}.

Figure 2 shows the resonance Raman spectra of the metcyano, carbonmonoxy, and oxyHbI heme-complex derivatives. The spectra also reveal an interesting feature: the absence of the propionate vibrational normal mode at 370 cm^{-1}. The unassigned small band at 390 cm^{-1}, present in the carbonmonoxy spectrum, is not related to the motion of the propionate due to its high-frequency value. Our results show that in HbI heme the presence of the propionate vibrational mode is insensitive to the oxidation state of the heme iron, but sensitive to the ligation state of the chromophore. For example, the 370 cm^{-1} band is weakly present in the metaquo and deoxyHbI heme derivatives, while absent in carbonmonoxy, metcyano, and oxyHbI heme ligand complexes.

Figure 3 shows the ^1H NMR spectrum of the paramagnetic metcyanoHbI (metHbICN) complex in H_2O at 19.7 °C. Heme methyl signals, 1-CH_3 at 17.86 ppm, 5-CH_3 at 25.73 ppm, and 8-CH_3 at 13.34 ppm, are identified. In a recent work,[7] heme methyl signals and heme-2-vinyl signals were assigned. The spectrum also shows in the resolved low field region, a single proton nonexchangeable resonance line at 17.90 ppm. This

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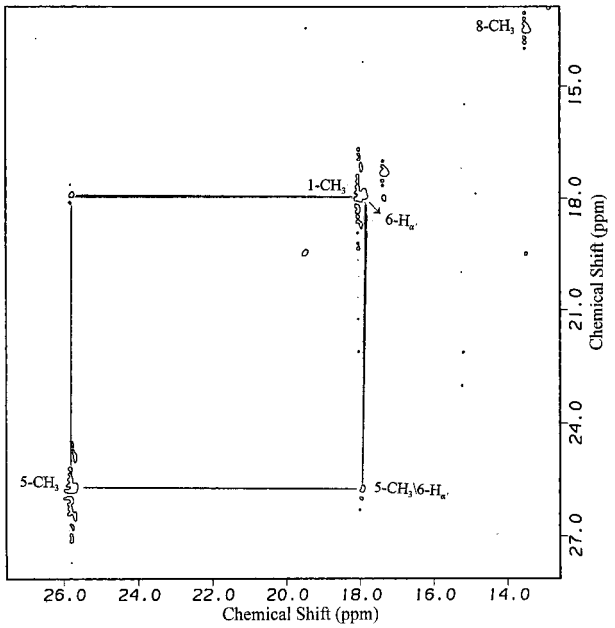

Figure 4. Downfield portion of the 600 MHz NOESY spectrum of the paramagnetic metHbICN complex in D_2O, pH 7.8, at 19.7 °C. The labeled cross-peak shows the connectivity between 5-CH_3 and 6-$H_{\alpha'}$ proton.

signal at 17.90 ppm presents a NOESY cross-peak (Figure 4) with the 5-methyl heme substituent (5-CH_3) at 25.73 ppm. The resonance lines at 17.90 ppm and 25.73 ppm (5-CH_3) are correlated to a high field signal at 5.82 ppm. The protons that produce the signal at 17.90 and 5.82 ppm must be next to 5-CH_3. The resonance line at 17.90 ppm is in the extreme low field region. This suggests, similar to other hemoglobins,[15,19−21] that the group involved can be a heme substituent or a heme pocket amino acid. On this regard, HbI possesses Phe43 (CD1) amino acid residue in the heme pocket, located between the 4- and 5-positions of the heme. Either some CD1 protons or the α protons from the 6-heme propionate group (6-H_α protons) can present dipolar coupling with 5-CH_3.

The observed chemical shifts for a proton on a paramagnetic complex can be regarded as the sum of paramagnetic and diamagnetic components. The paramagnetic shifts vary primarily as the inverse of the temperature. Ideally, the extrapolation to infinite temperature in the plot of observed chemical shifts versus the inverse of the absolute temperature (Curie plot) gives rise to the diamagnetic chemical shifts.[20] *T* dependence of chemical shifts for the metHbICN complex was measured and the chemical shifts at infinite temperature for the resonance line at 17.90 ppm (Figure 5) were extrapolated to 0.25 ppm. This value suggests that the signal is not from an aromatic proton. The region for diamagnetic aromatic protons is from 5 to 8 ppm.[20] The signal can originate from an aliphatic proton as 6-H_α. In previous studies, a signal with a similar extrapolated diamagnetic chemical shift for the CD1 C_ζH proton of the SW metMbCN proton was reported at 0.2 ppm.[20] Recent studies[6] on the metcyanoHbI complex have assigned the C_ζH protons of the Phe43(CD1) group at 7.26 ppm. These data together with our results from the *T*-dependent and the inversion−recovery experiments of the metcyanoHbI complex suggest that the 17.90 ppm signal corresponds to the 6-$H_{\alpha'}$ propionate proton.

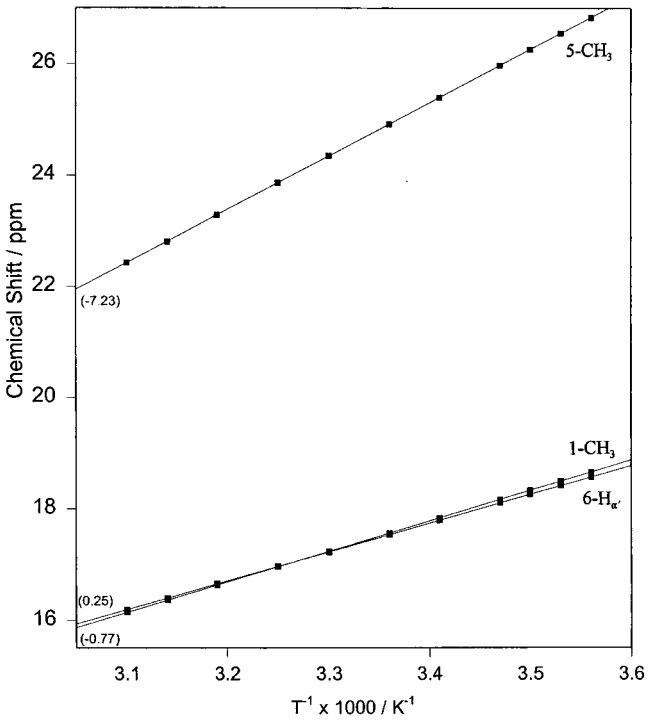

Figure 5. Curie plot of the observed shifts vs reciprocal of the absolute temperature for the met-HbICN hyperfine shifted resonances in the downfield region. Chemical shifts were obtained from 281 to 323 K (8 to 50 °C) in a Varian 300 MHz spectrometer. The numbers in parentheses correspond to the extrapolated chemical shifts at infinite temperature (T^{-1}) corresponding to the diamagnetic chemical shift of the protons for the diamagnetic form of the protein.

Additional information was obtained through T_1 measurements which also suggest that the 17.90 ppm signal is a propionate proton. The relaxation time is inversely proportional to the sixth power of the distance from a particular proton to the ferric iron center. According to X-ray studies[3,4] the nearest carbon (C_ζ) distance to the iron center for the CD1 residue in HbI corresponds to 4.92 Å. Assuming an average C−H distance, the C_ζH proton may be located ca. 4.11 Å from the heme iron. Similarly, the 6-$H_{\alpha'}$ propionate proton can be estimated at 5.79 Å from the iron center. In SW Mb[22] the CD1 C_ζH proton distance from the heme center is 4.49 Å and presents a T_1 less than 20 ms.[23] Thus, a similar value can be expected for the CD1 C_ζH proton in HbI. The shorter distance from 1-CH_3 group to the iron is 6.06 Å and the distance from the 6-$H_{\alpha'}$ group to the iron is at 5.79 Å. These data suggest that T_1 for both signals should be similar. Regarding this, the longitudinal relaxation time experiments revealed that the signal at 17.90 ppm relaxes similarly to 1-CH_3 with 97 ∓ 2 ms. Therefore the relaxation time determined for the resonance at 17.90 ppm in the metHbICN does not correspond to that for the CD1 C_ζH proton with an expected T_1 in the order of 20 ms. Furthermore, the distance of 6-$H_{\alpha'}$ propionate proton to 5-CH_3 is 2.41 Å, while the nearest nonring proton from CD1 to 5-CH_3 is 3.10 Å. This proton, located at 8.66 Å from the iron center, should have a paramagnetic chemical shift different from that of the 6-$H_{\alpha'}$ proton. Thus, the cross-peak shown in Figure 4 can be assigned to the interaction between 5-CH_3 protons and the 6-$H_{\alpha'}$ proton of HbI. These data also suggested that the signal at 17.90 ppm can be assigned to the 6-$H_{\alpha'}$ proton of the HbI heme propionate group.

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Discussion

Resonance Raman spectra of heme proteins display a vibrational normal mode attributed to the heme propionates. This band is usually found around 366 to 376 cm^{-1}, and it is assigned to the heme-6- and heme-7-propionate in-plane bending vibration.[16] This frequency has been correlated with the hydrogen bond strength between the heme-7-propionate and the nearby amino acid residues.[24] In most heme proteins the heme-6-propionate group hydrogen bonds to Lys or Arg in the CD3 position.[8a,b,9a,b,11,25] In horse heart Mb (HH Mb) a hydrogen bond network is formed between Leu 89, Ser 92, His 93, His 97, and the peripheral groups of the heme propionates. The heme-7-propionate hydrogen bonds to His 97 and Ser 92, while the heme-6-propionate is stabilized through hydrogen bonding with Lys 45.[12,26] Disruption of the hydrogen-bonding network between the heme-7-propionate and the nearby amino acid residues (His93, His97, and Ser92) lowers the frequency of the propionate band from 372 to 364 cm^{-1}. It has been suggested that a frequency of 372 cm^{-1} is indicative of a strong hydrogen bond, while a low frequency (below 369 cm^{-1}) is related to a weak hydrogen bond to the heme-7-propionate. This weak hydrogen bond could be formed with water molecules in the hydration shell of the heme environment.[27]

In HbI from *Lucina pectinata*, the propionate low-frequency vibration at 370 cm^{-1} is consistent with a moderate hydrogen bond between Arg99 and the heme-7-propionate of HbI. For other heme proteins, the absence of a moderate intensity of the propionate normal mode vibration from the 366−376 cm^{-1} region has been used by Friedman and Peterson[27] to explain a unique environment for the out-of-plane heme-7-propionate. The heme-7-propionate of HH Mb is involved in a hydrogen bonding network,[12,13,26] which HbI lacks, as indicated by this work and X-ray crystallography[3,4] of a strong hydrogen bonding network (see Figure 6). The heme-7-propionate forms a hydrogen bond,[3] different from HH Mb, only with Arg(99)FG2. Furthermore, the disappearance of the small intensity propionate band upon formation of the metcyano, carbonmonoxy, and oxyHbI heme-complex derivatives suggests a change in the environment around the propionate group when the six-coordinated HbI moiety is formed. Regarding this, *Nassa mutabilis* Mb has a small intensity propionate band at 378 cm^{-1}, a behavior attributed to a different interaction of the protein with the propionate groups.[17] The RR data suggest a model where the heme group of HbI in *Lucina pectinata* is tightly bound to His96 (ν_{Fe-His} at 218 cm^{-1}). However, due to the absence of strong hydrogen bonding interactions between the heme propionates and the nearby amino acids, the heme group is not firmly anchored. Changes in the strength of the hydrogen bonding from moderate to weak are observed upon formation, as indicated by the disappearance of the 370 cm^{-1} band of the HbI heme-ligand moieties. These two observations suggested a flexible heme rocking motion that contributes to the HbI ligand binding mechanism.

The heme-6-propionate α protons have been located in the downfield region, in most cases in the resolved portion of the spectrum.[15,19,22] The resonance line for the α protons of the heme-6-propionate was detected in the ^1H NMR spectrum of the metcyanoHbI complex at 17.90 ppm. The longitudinal relaxation time experiments and NOESY spectrum of the paramagnetic metHbICN complex also indicated that the signal at 17.90 ppm can be assigned to the 6-$H_{\alpha'}$ proton of the HbI heme propionate group. Table 1 summarizes the chemical shifts for the heme-6-propionate α protons of metcyano complexes of elephant Mb (El metMbCN),[15] sperm whale Mb (SW metMbCN),[15] and *Lucina pectina* HbI. The ^1H NMR data for the heme-6-H_α protons in metcyanoHbI are very different in comparison to the metcyano complexes of elephant Mb and sperm whale Mb. Variations in chemical shifts for the heme-6-propionate α protons, when compared to SW Mb and El Mb, are in the order of 0.81 to 10.51 ppm. These large differences in chemical shifts suggest a lack of hydrogen bonds between the heme-6-propionate group and the amino acid residues from the polypeptide chain. Our data are consistent with the X-ray data[3] where the CD3 residue is the apolar residue Leu 46, and no hydrogen bond is expected for the heme-6-propionate. Figure 6 shows such an environment. Similar differences in chemical shifts have been observed for the 6-propionate protons of *Rhodomicrobium vannielli* cytochrome c_2, lacking the Arg residue in the CD3 position, and *Rhodopseudomonas viridis* cytochrome c_2, having the Arg residue.[25] In HbI structural overlays bring Leu(46)CD3 in almost exact coincidence with the side chain of Arg(45)CD3 in sperm whale Mb.[3] Thus, the absence of hydrogen bonds for the HbI heme-6-propionate may predict spectral differences between these heme-proteins. We suggest that the difference in chemical shift of the α protons of the heme-6-propionate group in HbI is due to the lack of

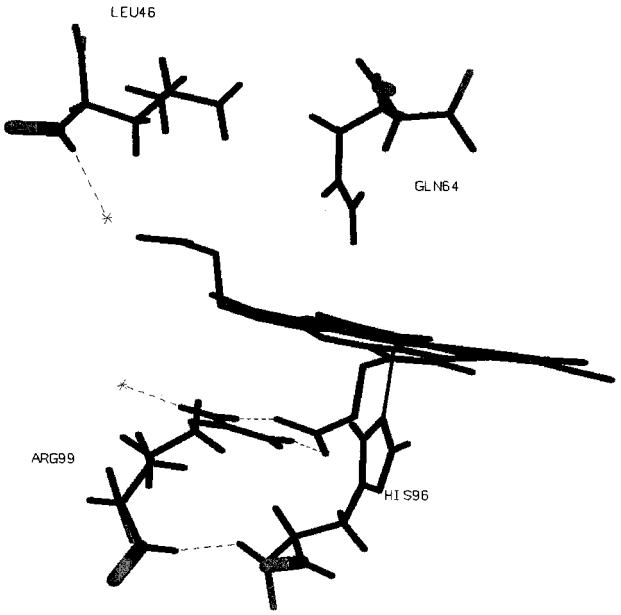

Figure 6. Hydrogen bonding environment for the heme propionates in HbI. The heme-7-propionate forms a weak to moderate hydrogen bond only with Arg(99)FG2 as indicated by this work and X-ray crystallography.[3,4]

Table 1. Chemical Shifts (ppm) for 6-H_α Protons in El Mb, SW Mb, and HbI Metcyano Complexes

proton	SW metMbCN[a]	El metMbCN[a]	metHbICN[b]
6-H_α	9.20	6.63	5.82
6-$H_{\alpha'}$	7.39	10.30	17.90

[a] From ref 15 (obtained at 30 °C and pH 8.6). [b] Our results (obtained at 19.7 °C and pH 7.8).

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hydrogen bond with the residue in the CD3 position due to its apolar nature and to a flexible orientation of the side chain with respect to the heme plane. Therefore, the heme-6-propionate does not contribute to anchor the heme group with the polypeptide chain of HbI. This flexibility contributes to the proposed rocking motion of the HbI chromophore.

^{1}H NMR spectroscopy also has been used to study the heme-7-propionate α protons in proteins. The chemical shifts for the heme-7-propionate α protons have been found in the diamagnetic envelope of the metcyano complex of other hemoglobins,[15,22] but in some cases it has not been determined.[19] This is the case for the 7-propionate α protons of the metcyanoHbI complex. For horse heart myoglobin, the heme-7-propionate hydrogen bonds to Ser92. Site directed mutagenesis of horse heart myoglobin was made and the Ser92Asp variant was obtained.[12] ^{1}H NMR results for this myoglobin mutant, in comparison to the wild-type Mb, showed variations in the chemical shifts of the α protons of the heme-7-propionates. These variations were interpreted in terms of a reorientation of the propionate side chain and the loss of the hydrogen bond between the Ser residue and the heme-7-propionate group.

Conclusion

The spectral data presented here suggest a model where the heme group in HbI from *Lucina pectinata* is tightly bound to His96 (ν_{Fe-His} at 218 cm^{-1}) as in El Mb,[18] HH Mb,[12,26] and SW Mb,[9,10] but, due to the very weak 370 cm^{-1} propionate normal mode, the heme group is not firmly anchored to the polypeptide chain. This conclusion also is supported by our ^{1}H NMR results. Furthermore, the disappearance of the 370 cm^{-1} propionate vibration suggested changes on the hydrogen bonding strength of the environment of heme propionates and the nearby amino acids upon ligand binding. This behavior can facilitate a heme rocking freedom that promotes the bonding between HbI and the in-coming ligand.

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