Confirmation of the Assignment of the Iron–Histidine Stretching Mode in Myoglobin

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Abstract: Resonance Raman spectra of myoglobins reconstituted with hemes isotopically substituted at the central iron atom or the pyrrole nitrogen atoms have been recorded to address the issue of whether the strong line at ~220 cm^{-1} is the iron–histidine stretching mode or the iron–pyrrole nitrogen stretching mode. The frequency of the line at 220 cm^{-1} is 1.7 cm^{-1} lower in myoglobin reconstituted with the ^{57}Fe heme than it is in the ^{54}Fe-substituted heme. No large shifts were detected in any other Raman lines. When myoglobin reconstituted with ^{15}N-substituted pyrrole nitrogens in the heme is compared to the unsubstituted myoglobin no large change is detected in the line at 220 cm^{-1}, but the frequency of the line at 243 cm^{-1} is 1.5 cm^{-1} lower. In comparing myoglobin buffered in D_2O to that buffered in H_2O only the line at 220 cm^{-1} changes frequency (1.4 cm^{-1}). From these isotopic substitution studies, we conclude that the line at ~220 cm^{-1} in myoglobin is the iron–histidine stretching mode. The mode at ~243 cm^{-1} has a significant contribution from the pyrrole nitrogens, and it is likely an out-of-plane pyrrole tilting mode. The ^{54}Fe–^{57}Fe isotope shift of 1.7 cm^{-1} in the 220-cm^{-1} line is smaller than predicted for a diatom oscillator of the iron and the histidine. We conclude that the iron–histidine stretching mode is either mixed with an internal mode of the histidine and/or mixed with skeletal modes of the porphyrin macrocycle.

Resonance Raman scattering has been applied extensively to characterize the structure of heme proteins. The goal of these studies is to determine the relationship between the protein control of electronic and molecular structure at the heme and the physiological properties of the macromolecule. In order to fully utilize the potential of resonance Raman scattering, it is essential to assign the normal modes of the porphyrin complex. Of particular interest are the vibrations involving the iron–histidine (Fe–His) bond in heme proteins since in hemes *a* and *b* this is the only covalent linkage to the protein. In hemoglobin it has been proposed that protein structural changes in response to ligand binding may be mediated through this bond.[1-3]

The assignment of the Fe–His stretching mode is controversial in deoxy Hb and Mb. From work in several laboratories[4-7] the line in the 200–230-cm^{-1} region has been assigned as the Fe–His stretching mode. The main evidence supporting this assignment is the following: 1. Isotopic substitution of the iron atom has been reported to give the theoretically expected shift in the 220-cm^{-1} line in Hb[6], Mb[4], and model compounds.[5] 2. Deuteration of the imidazole in model compounds also yields a significant isotope shift in the 220-cm^{-1} line.[5,7] 3. In contrast to pentacoordinate model compounds, in tetracoordinate compounds the 220-cm^{-1} line is absent but other Raman lines are relatively unaffected,[6] suggesting that the 220-cm^{-1} line originates from the 5th ligand. 4. The porphyrin modes in picket fence model compounds differ from those of protoporphyrin, but the 220-cm^{-1} line is at the same frequency, when the 5th ligand is 2-methylimidazole.[5] 5. In horse radish peroxidase the pH dependence of the Raman scattering is limited to the 220–250-cm^{-1} region.[8] The labile group in this protein is presumed to be the proximal histidine. 6. In low-temperature resonance Raman spectra of hemoglobin, it is observed that the 220-cm^{-1} line is completely absent with 457.9-nm excitation but present with 441.6-nm excitation. This was attributed to a charge-transfer intermediate state for enhancement of the Fe–His vibration in contrast to porphyrin π–π^* transitions which enhance porphyrin modes.[9]

In contrast, from work in another laboratory the Fe–His stretching mode was assigned at 408 cm^{-1} and the 220-cm^{-1} line was assigned as a iron–pyrrole nitrogen stretching mode.[10-15] The evidence cited supporting the assignment of the 220-cm^{-1} line as the iron–pyrrole nitrogen stretching mode is the following: 1. The mode at 220 cm^{-1} was observed to shift to lower frequency on isotopic (^{15}N) substitution of the pyrrole nitrogens in a model compound.[12] 2. There is an inverse correlation between the iron-to-pyrrole nitrogen distance and the frequency of the 220-cm^{-1} line. As the iron-to-pyrrole nitrogen distance increases, the frequency of the line decreases.[15] 3. There is a correlation between the frequency of the line in the 220-cm^{-1} region and the iron-to-heme plane distance. Higher frequencies correspond to the iron being closer to the heme plane. A simple analysis was made showing that this could be consistent with an iron–pyrrole nitrogen mode assignment.[14] 4. Finally, a negative correlation was detected between the pK_a of the axial ligand and the frequency of the 200–230-cm^{-1} line. It was argued that the frequency of the Fe–His bond should increase with the increase in pK_a, and this increased strength of the axial bond should weaken the iron–pyrrole nitrogen bonds.[13]

In order to clarify this assignment we have carried out new experiments on isotopically labeled myoglobin. With isotopic substitution of the iron or pyrrole nitrogen, only small frequency shifts (≈1 cm^{-1}) are expected for most normal modes because of the small percentage changes in the reduced masses. Conventional

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Raman measurements are not sensitive enough to define small shifts with a high reliability. However, small frequency shifts can be detected with Raman difference spectroscopy.[16] In this technique, the problem of detecting a small frequency shift in an isolated Raman line is reduced to the determination of the intensity in the difference spectrum which may be measured quite accurately if a good signal-to-noise ratio is obtained. Systematic errors due to spectrometer resetability are eliminated, and under favorable conditions frequency shifts of less than 0.1 cm^{-1} can be detected reliably.

We have utilized Raman difference spectroscopy to accurately determine shifts in Raman lines of Mb isotopically labeled at the iron and at the pyrrole nitrogens. It is shown that the 220-cm^{-1} line is primarily due to the Fe–His stretching mode and the 243-cm^{-1} line has a contribution from motion of the pyrrole nitrogens.

Materials and Methods

For reconstitution of ^{15}N into the pyrrole rings of the myoglobin, isotopically substituted heme *b* was prepared by growing *Rhodopseudomonas spheroides* on enriched amino acids as described in Scholes et al.,[17] leading to 91.8% total ^{15}N enrichment. The samples used for the ^{14}N–^{15}N comparison were also ^{57}Fe (90%) enriched according to the methods of Yonetani and Asakura,[18] Inubushi and Yonetani,[19] and Teale,[20] so that ENDOR measurements could be made on the same material.[17] Additional ^{57}Fe or ^{54}Fe isotopically substituted myoglobin samples were prepared by inserting the isotopically substituted hemin (Porphyrin Products) into the apomyoglobin prepared according to the method of Teale.[20] Optical absorption spectra confirmed that the native heme was completely removed from the apomyoglobin. Spark source mass spectral analysis of the hemin was made to ascertain the isotopic purity of the iron. The ^{54}Fe sample was found to be 97% enriched and the ^{57}Fe sample was 91% enriched. Optical absorption spectra were used to confirm that the reconstituted myoglobins were structurally the same as the native preparations. Purified native myoglobin [(^{56}Fe–^{14}N) Mb] was donated by Dr. Frank G. Gurd of the Indiana University. Mb preparations were dissolved in 100 mM phosphate buffer, pH 7.0, at the concentration of 50 μM. Immediately prior to running the spectra, the samples were passed through a Sephadex G-25 column and a minimal amount of sodium dithionite was added to reduce the Mb in a N_2 atmosphere. The samples were then introduced into the difference spinning cell and sealed under N_2.

Raman difference spectra were recorded with the instrumentation described by Rousseau.[16] The excitation source was the 441.6-nm line of a He–Cd laser. The spectrometer step size was 0.4 cm^{-1}, and the spectral band pass was 5 cm^{-1}. The spectra were signal averaged about 50 times to achieve the high signal-to-noise ratio needed for the determination of reliable frequency differences. Before and after recording difference spectra of myoglobins, both compartments of the spinning cell were filled with carbon tetrachloride and spectra were recorded for calibration of the spectrometer by using the apparent shift in the 218-, 314-, and 459-cm^{-1} lines. This also gave the instrumental limitation for detecting a shift between samples in the two compartments, which was found to be less than 0.1 cm^{-1}. Actual frequency differences which are detected have somewhat larger errors owing to a lower signal-to-noise ratio in our myoglobin samples than in the organic solvent samples.

All the spectra are presented without smoothing. The oxidation state marker band at 1356 cm^{-1} was monitored before and after recording the low-fequency spectra in order to ascertain sample integrity.

Results

Figure 1 shows the low-frequency Raman spectra and the Raman difference spectrum of ^{54}Fe Mb and ^{57}Fe Mb. In the difference spectrum a characteristic difference shape is observed at 220 cm^{-1} which represents a 1.5 ± 0.2 cm^{-1} shift to lower frequency in the ^{57}Fe isotopically labeled sample. From several spectra we find that the average ^{54}Fe–^{57}Fe shift is 1.7 ± 0.2 cm^{-1}. The shift in this line is consistent with but somewhat smaller than the isotopic shifts in this line reported in hemoglobin,[6] myoglobin,[4] and porphyrin model compounds[5] with Fe isotope substitution by other workers which were done without the Raman difference apparatus. Apart from the changes in the line at 220 cm^{-1} and from the very small changes evident in a few other lines in Figure 1, no changes were detected in any other lines in 150–1700-cm^{-1} region. Any possible iron isotope frequency difference in the line at 242 cm^{-1} is estimated to be less than 0.4 cm^{-1}.

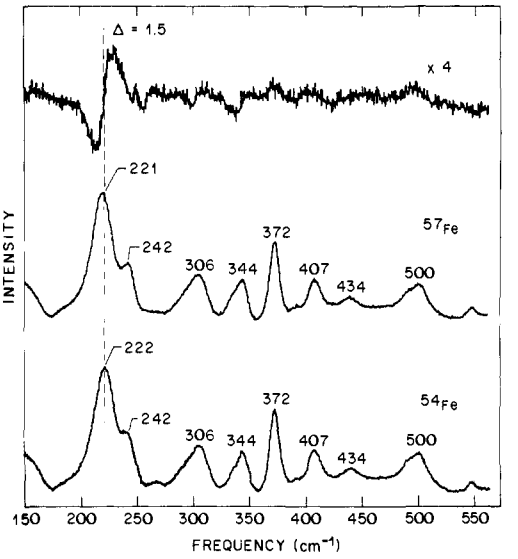

Figure 1. Raman spectrum of ^{54}Fe Mb and ^{57}Fe Mb recorded on the Raman difference apparatus. Both of the samples were 50 μM in phosphate buffer (100 mM, pH 7.0). The laser excitation wavelength was 441.6 nm (He–Cd laser), and the power was 20 mW. The spectrometer step size was 0.4 cm^{-1}, and the spectral band pass was 5 cm^{-1}. The spectra were signal averaged for 50 scans (16 h). The scale for the intensity in the difference spectrum has four times the sensitivity as that of the individual Raman spectra. The Δ values on the difference spectrum are shifts to the lower frequency in cm^{-1} of the indicated lines calculated from the difference spectrum. The error in determining the peak positions is ±0.5 cm^{-1} whereas that in determining the shift in the line from the difference spectrum is ±0.2 cm^{-1}.

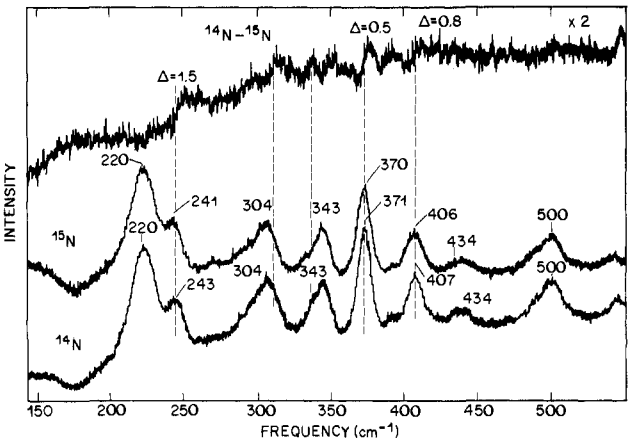

Figure 2. Raman spectrum of (^{57}Fe–$^{14}N_4$) Mb and (^{57}Fe–$^{15}N_4$) Mb. The difference spectrum (at twice the sensitivity of the Raman spectra) was obtained by subtracting the latter from the former. All the conditions are the same as in Figure 1.

Figure 2 shows the low-frequency Raman spectra and the difference spectrum of ^{57}Fe–$^{14}N_4$ and ^{57}Fe–$^{15}N_4$ substituted hemes in Mb. The difference spectrum shows a 1.5 ± 0.4 cm^{-1} shift in the line at 243 cm^{-1}, a 0.5 ± 0.2 cm^{-1} shift in the line at 372 cm^{-1}, and a 0.8 ± 0.4 cm^{-1} shift in 407-cm^{-1} line. Changes in shoulders on lines at 304 and 343 cm^{-1} are also evident in the spectra. Any possible pyrrole nitrogen isotope frequency shift in the line at 220 cm^{-1} is less than 0.4 cm^{-1}. In the higher frequency region (data not shown) significant differences were also detected. The largest changes were a 3.4 ± 0.1 cm^{-1} shift in the line at 672 cm^{-1} and

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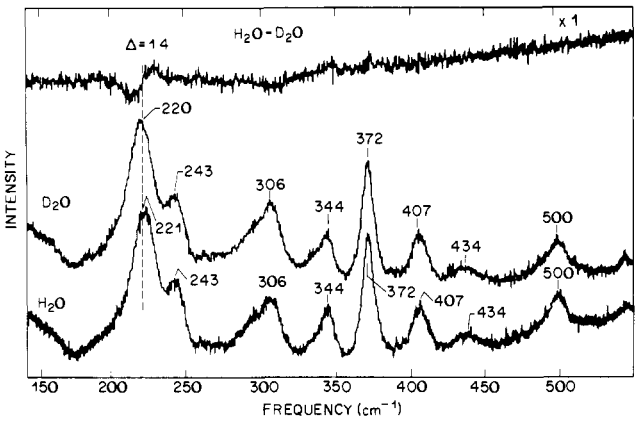

Figure 3. Raman spectrum of Mb in phosphate buffer (100 mM, pH 7.0) vs. Mb in D_2O phosphate buffer (100 mM, pD 7.3). The difference spectrum (at the same sensitivity as the Raman spectra) was obtained by subtracting the latter from the former. All the other conditions are the same as in Figure 1, except the spectra were signal averaged for 41 scans (13 h).

4.7 ± 0.1 cm^{-1} in the line at 1356 cm^{-1}.

The spectra of deoxy Mb in H_2O phosphate buffer and deoxy Mb in D_2O phosphate buffer are shown in Figure 3. As is evident in the difference spectrum there is a 1.4 ± 0.4 cm^{-1} shift to lower frequency in the line at 220 cm^{-1} only. No shift in frequency of any other line in the spectrum was detected.

Discussion

The correct assignment of the line at 220 cm^{-1} is of pivotal importance in understanding the molecular basis of the heme protein function since it displays a wide range of frequencies depending on the protein properties. Furthermore, the question of whether it is the Fe–His stretching mode or an Fe–N (pyrrole) stretching mode must be settled to allow a quantitative determination of possible cooperative energy storage at the heme in hemoglobin. From the new data reported here we can now definitively assign the line at ~220 cm^{-1} in Mb as the Fe–His stretching mode. Thus, our results confirm the assignment made by Kitagawa and co-workers[4-6,8] as well as by Kincaid et al.[7] and are at variance with the conclusions drawn by Desbois et al.[10-15]

Calculations assuming a diatomic Fe–His oscillator predict a ^{54}Fe–^{57}Fe isotope shift of 3.2 cm^{-1}. The observed shift in the 220-cm^{-1} line in Mb is 1.7 cm^{-1} for this isotopic substitution. It is the only line with a large iron isotope frequency shift in the spectrum. Previously, isotope shifts for the line in the 220-cm^{-1} region have been reported for Hb[6], Mb[4], model compounds,[5] and most recently cytochrome oxidase.[21] The observation that this is the only line with iron isotope sensitivity is consistent with its assignment as the iron–histidine stretching mode when considering its polarization characteristics. The iron–histidine stretching mode preserves the full symmetry of the five-coordinate nonplanar heme and hence should be fully polarized. The only other possible polarized out-of-plane mode involving the iron would have the iron moving in phase with the histidine through the porphyrin plane. Although the iron isotope shift depends on the number of porphyrin atoms which contribute to this mode, a shift of 1–2.5 cm^{-1} for the ^{54}Fe–^{57}Fe comparison is not unreasonable and is consistent with our observed shift. However, normal mode calculations[22] indicate that the frequency of such a mode should be very low (<100 cm^{-1}). Thus, we can rule out this mode as the origin of the line at 220 cm^{-1}. In-plane modes involving the iron will not be fully polarized as the mode at 220 cm^{-1} is observed to be. Thus, the observed isotope dependence of a fully polarized mode is consistent only with an out-of-plane mode strongly involving the iron. We observe an isotope shift of 1.7 cm^{-1}, smaller than the calculated shift of 3.2 cm^{-1}, assuming the iron and the imidazole as a diatom pair. This indicates that the iron–histidine stretching motion is coupled to either an internal motion of the imidazole and/or a motion involving atoms of the porphyrin macrocycle.

On placing the deoxy Mb in buffered D_2O the line at 220 cm^{-1} is the *only* line to change frequency. If the 220-cm^{-1} line had Fe–N (pyrrole) character, other low-frequency lines would be expected to change since the isotopic data as well as normal mode calculations[22] show that the pyrrole nitrogens are involved in many of the low-frequency modes. The frequency shift of *only* the mode at 220 cm^{-1} is consistent with it being the Fe–His stretching mode since a change at the imidazole due to deuteration of exchangeable protons could affect only those modes involving the imidazole, not others. Our observation of a shift of 1.4 cm^{-1} on deuteration is significantly *larger* than that predicted (0.75 cm^{-1}) on the basis of the exchange of one labile proton in a diatomic oscillator (Fe vs. imidazole). The change that we detect is therefore most consistent with a protein structural change due to deuteration of labile protons on other amino acids of the protein. Such an exchange could slightly change the structural relationship between the heme and the histidine resulting in a larger than expected shift.

From Figure 2, it is apparent that there is no large shift in the 220-cm^{-1} line upon ^{15}N isotope substitution of the pyrrole nitrogens. This rules out a normal coordinate contribution from these atoms to the mode at 220 cm^{-1}. This is consistent with the assignment of this line to the Fe–His stretching motion which would be expected to have only a small contribution from the four pyrrole nitrogen atoms. On the other hand, an iron–pyrrole nitrogen stretching mode should have a large contribution from these atoms. In addition to a ^{15}N isotope sensitivity in several other modes (see Figure 2), the 243-cm^{-1} line displays a significant ^{15}N isotope dependence.

The origin of the 243-cm^{-1} line in the Raman spectrum of Mb is yet to be understood. Even though there is a one-to-one correspondence for most lines in the low-frequency region of deoxy Mb and Hb, a counterpart of the 243-cm^{-1} line in the former is absent in the latter. It has been argued[6] that an $Fe-N_{pyrrole}$ stretching vibration may be very close in frequency to the Fe–His stretching vibration. Since we have assigned the 220-cm^{-1} line to the Fe–His stretching mode, we have investigated the possibility that the 243-cm^{-1} line in Mb is the $Fe-N_{pyrrole}$ stretching mode. The 1.5-cm^{-1} frequency shift detected in this mode upon ^{15}N (pyrrole) substitution is consistent with this idea. However, isotopically substituted substituent (methyl) groups also result in frequency shifts in the 243-cm^{-1} mode.[23] Therefore, this mode is not a simple Fe–N (pyrrole) mode but contains contributions from many atoms. Its sensitivity both to the mass of the pyrrole nitrogen atoms as well as to peripheral substituent groups make it a good candidate for an out-of-plane pyrrole ring-folding mode. Choi and Spiro recently found evidence for such modes, in this frequency region.[24] The counterpart for this mode in Hb and other heme proteins remains to be determined.

The isotope substitution data on Mb reported here do not agree with those reported by Desbois et al.[10-15] on Mb and on model compounds. They had assigned the 408-cm^{-1} line as the Fe–His stretching mode. We do not attribute any Fe character to the 408-cm^{-1} line since it shows no significant iron isotope shift. In addition, contrary to the observation of a 1.5-cm^{-1} shift in the 501-cm^{-1} line by Desbois et al.[10,11] upon ^{54}Fe substitution, we do not find any iron isotope shift in this line either. Finally, the strongest evidence favoring the assignment of the 220-cm^{-1} line as being a mode involving the pyrrole nitrogens has been the reported ^{15}N (pyrrole) isotope dependence of this line in model compounds.[12] We do not detect a significant frequency shift in the 220-cm^{-1} line upon ^{15}N substitution in Mb. We note that Desbois et al.[10-15] did not use a Raman difference apparatus to measure their differences as we have done. We propose that the differences between our isotope substitution studies and theirs is a consequence of the difficulties in trying to detect very small differences without Raman difference instrumentation. However,

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a systematic difference between the behavior of the Mb and the model compounds cannot be excluded.

As the isotope dependence is incontrovertible, it is necessary to account for the additional points cited by Desbois et al.[10-15] supporting their assignment of the line at 220 cm^{-1} being the iron–pyrrole nitrogen mode. They observed an inverse correlation between the 220-cm^{-1} line and the iron–pyrrole nitrogen distance[15] and the iron-to-heme plane distance.[14] An inverse correlation was also found for the frequency of this line and the iron–nitrogen (histidine) distance[15] although there was significantly more scatter in the data for this latter correlation. It is our contention that a correlation between the 220-cm^{-1} line and any of these three distances does not prove the mode assignment and may even be very disceptive. For example, consider the emperical correlation between the core size (pyrrole nitrogen to heme center distance) and several of the high-frequency porphyrin skeletal modes.[25] These modes involve motion of the meso carbon atoms of the porphyrin macrocycle, but their frequencies are sensitive to the heme center structure. This sensitivity to heme expansion or contraction results from the overall porphyrin distortion being accommodated by the bonds of the meso carbon atoms due to the rigidity of the pyrrole rings. Thus, it is not appropriate to cite correlations as evidence for normal coordinate distribution. In the case of the correlations between the 220-cm^{-1} line and these measured distances, it must be kept in mind that these three distances [Fe–N_{His}, Fe–$N_{pyrrole}$, Fe–Ct] would not be expected to change independently. For example, as increased protein forces or steric interactions in model compounds cause elongation of the iron–histidine bond, concomitant changes in the iron–pyrrole nitrogen and iron out-of-plane distance would be expected. Thus, as the Fe–His bond is strained, the Fe–His stretching frequency is lowered since the bond is weakened, but at the same time the iron would be expected to move further out of plane and lengthen the Fe–pyrrole nitrogen bond distance accounting for all of the correlated data.

Finally, the pK_a dependence[13] presents a more complex situation. Considering pK_a effects alone, the Fe–His bond strength should increase as the pK_a of the base increases. The effects of the hindering groups must also be considered, and for the most part, for the compounds studied they should tend to cancel the pK_a effects. However, from equilibrium measurements, it has been reported that they do not bring about a cancellation and the ligand binding affinity with hindered imidazoles is somewhat *greater* than that of unhindered imidazoles.[26-28] This is a very surprising observation as one would expect the steric interactions to dominate. In more recent equilibrium measurements, however, systematic trends of decreased ligand binding affinities with increased steric hinderance have been reported.[29] The dominance of the steric interactions is supported by the Raman measurements. The more hindered imidazoles have lower frequencies than do the less hindered imidazoles. However, 1-methylimidazole, which is not hindered, would be expected to follow the pK_a and have a higher frequency than imidazole. A significantly lower frequency is observed (210 cm^{-1} for 1-methylimidazole vs. 223 cm^{-1} for imidazole).[13] A similar relationship exists between 2-methylimidazole (205 cm^{-1}) and 1.2-dimethylimidazole (194 cm^{-1}).[13] We speculate that this results from either changes in π-bonding character due to adding the methyl groups at the imidazole nitrogen or the fact that the normal mode is altered by the substitution.

In conclusion, the data presented here firmly establish the line at 220 cm^{-1} in myoglobin as the Fe–His stretching mode. By analogy in all heme *a* and *b* containing proteins with histidine as the 5th ligand, the Fe–His stretching mode is expected to occur in the 220-cm^{-1} region. Detailed normal mode assignments are still needed to determine which factors cause frequency variations in this mode since it shifts substantially as structural properties of heme proteins are varied. Furthermore, it has been observed recently that there is a correlation between the 220-cm^{-1} line and the equilibrium constant for binding the first dioxygen molecule in hemoglobin,[30] thereby making it even more imperative that the spectroscopic properties of the Fe–His stretching mode are properly understood.

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Registry No. ^{54}Fe, 13982-24-6; ^{57}Fe, 14762-69-7; ^{15}N, 14390-96-6; Fe, 7439-89-6; His, 71-00-1.

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