Resonance Raman detection of structural dynamics at the active site in hemoglobin
(dephasing/heme/porphyrin vibrations)

M. R. Ondrias*, D. L. Rousseau*, and S. R. Simon†

*Bell Laboratories, Murray Hill, New Jersey 07974; and †State University of New York at Stony Brook, Stony Brook, New York 11794

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ABSTRACT The iron-histidine stretching mode in deoxygenated hemoglobin displays a large change in frequency and width upon lowering the temperature from 300 to 10 K. The temperature dependence of the data indicates the presence of dynamic processes. The dynamics of this mode in frozen hemoglobins can be qualitatively and quantitatively described as a vibrational dephasing via anharmonic coupling to other vibrations of the heme-imidazole system. The effects that occur at the melting transition in the low frequency modes cannot be quantitatively addressed at this point but may be indicative of the introduction of additional degrees of freedom predicated on protein influences that reflect differences in protein quaternary structure.

Large amplitude motions of atoms and groups of atoms have recently been detected in proteins by crystallographic (1, 2) and spectroscopic (3–5) techniques. Although in some instances the functional importance of these motions has not yet been assessed, in others the importance is clear. For example, the heme binding sites in hemoglobin and myoglobin are only accessible because molecular motions occur that modulate the size of the narrow entry channel and thus allow the penetration of small ligands (6). These motions involve both transitions between local minima in the potential energy surface (conformational substates) and thermal population of excited levels. A picture emerges of fluid-like proteins in which molecular groups rotate about single bonds, hydrogen bonds break and reform, and new Van der Waals interactions occur continuously.

There have been few reports in which dynamic processes in biomolecules have been probed by Raman scattering (7–10) although its potential for such studies is widely recognized because the Raman frequencies associated with bonds that connect molecular groupings are sensitive to structural dynamics. Resonance Raman scattering studies of dynamic processes in heme proteins are especially promising. The technique can be a sensitive probe of the properties of the active sites where the existence of structural dynamics could have direct functional importance.

We report here a resonance Raman investigation of hemoglobin in which we have detected a temperature-dependent variation in the width and position of the mode assigned as the iron-histidine (Fe-His) stretching motion. We interpret these results as evidence for structural dynamics involving fluctuations in the Fe-His bond energy due to anharmonic coupling between modes.

METHODS AND MATERIALS

Raman scattering data from solutions above and just below freezing were obtained with previously described Raman difference instrumentation (11). Samples were placed in glass or fused silica rotating cells and the scattered light was gathered with 90° collecting optics. For the low temperature studies (<−50°C), the rotating liquid cell was removed and liquid samples were placed on the cold finger of a Heli-Tran Cryostat (Air Products and Chemicals, Allentown, PA) where they were frozen either by direct insertion into liquid nitrogen (fast freeze) or a slow cooling of the cold finger (slow freeze). The samples were placed under vacuum and adjusted to the desired temperature down to about 10 K. We estimate an uncertainty of up to +10 K in our thermocouple temperature reading in the studies with the Heli-Tran due to local laser heating at the sample surface. These measurements were made in an approximately 180° geometry. All spectra were obtained with 4579 Å excitation from a Coherent Radiation model CR-18 argon ion laser and collected in 5 hr or less. Modified hemoglobins were prepared by the methods of Kilmartin and Hewitt (12) (NES-des-Arg-HbA) and Tyuma et al. (13) (α and β chains). Hemoglobin Kempsey was prepared according to Bun et al. (14). All samples were stored in liquid nitrogen, diluted with appropriate buffers, and deoxygenated by gentle agitation in a nitrogen atmosphere followed by the addition of a trace amount of sodium dithionite.

The Raman difference technique is well documented as a means of detecting small differences in the Raman lines of two slightly different samples (11, 15). It takes advantage of the fact that the intensity in the derivative-type difference spectrum is proportional to the frequency separation of the lines being compared. For very small frequency differences the limiting value of the frequency separation between the maximum and minimum in the difference spectrum is \(\Gamma/\sqrt{3}\) for Lorentzian lines and \(\Gamma/\sqrt{2}\ln 2\) for Gaussian lines where \(\Gamma\) is the full width at half height. Consequently, in the difference spectrum obtained by comparing a 20-cm\(^{-1}\) wide line of slightly different frequencies (<<\(\Gamma\)) in two samples, there is a substantial quantitative difference in the separation between the maximum and minimum depending on the functional shape of the line (11.5 cm\(^{-1}\) for Lorentzian lines and 17.0 cm\(^{-1}\) for Gaussian lines). To measure lineshapes on our Raman difference apparatus we store the same spectrum in two sets of memory locations in a minicomputer. One spectrum is then shifted in frequency relative to the other by a predetermined small amount (<<\(\Gamma\)) and a difference spectrum is generated. Although Raman lineshapes generally are a convolution of Gaussian and Lorentzian contributions, the separation between the maximum and minimum in the resulting spectrum allows determination of the predominant character of the lineshape (15).

RESULTS

The low frequency (\(\Delta\nu = 100–500\) cm\(^{-1}\)) spectra of hemoglobins exhibit several vibrational modes that display varying degrees of sensitivity to changes in quaternary structure (16, 17). A comparison of spectra obtained via Raman difference spectroscopy of the high affinity (R) and the low affinity (T) quater-
The quaternary structure of the protein is shown in Fig. 1. The difference spectrum displayed above the reference spectra is qualitatively representative of the differences observed for a broad range of R/T comparisons. The largest change induced by the quaternary structure transition occurs in the line in the 215–222 cm\(^{-1}\) region that has been assigned as the Fe-His stretching frequency (18). There are also changes in frequencies and intensities of other lines in the low frequency region (17) as may be seen from the structure in the difference spectrum. In addition there is an increase in intensity of the low frequency wing in T-structure hemoglobin.

The lineshapes of the Raman lines from deoxyhemoglobin in solution were measured by the methods described in the previous section, thus affording an accurate determination of the linewidth, position, and profile of the low frequency vibrations. This analysis shows that all the isolated lines in the Raman spectra of both the R and T forms of hemoglobin are predominantly Lorentzian in character except the lines at \(216\) and \(300\) cm\(^{-1}\), both of which are primarily Gaussian.

Low temperature spectra (77 K) were obtained for various hemoglobins and isolated chains to explore the origins of the broadening in the two low frequency modes. Typical spectra are shown in Fig. 2. For all of the hemoglobins that were examined nearly all of the low frequency—and hence thermally populated—lines narrow at low temperature. However, the two lines that are inhomogeneously broadened at room temperature behave quite differently at low temperature. The \(200\) cm\(^{-1}\) band remains quite broad, shifts to slightly higher frequency, and in some cases displays partially overlapping structural features. On the other hand, the Fe-His mode becomes Lorentzian and narrows to a width as small as any of the porphyrin modes (7–9 cm\(^{-1}\)). Moreover, it shifts by 10–15 cm\(^{-1}\) to higher frequency (229 cm\(^{-1}\)). Finally, the Fe-His stretching frequency and width, which were correlated with quaternary structure at room temperature, become identical for all forms of hemoglobin examined at 77 K.

A temperature dependence study of the Fe-His stretching mode was carried out to determine at what point the R/T distinguishability in the mode was lost. Fig. 3 shows representative spectra of HbA in dilute phosphate buffer from 77 to 275 K (solution) and demonstrates that upon warming, the Fe-His stretching mode continuously broadens and shifts to lower frequency, until at 270 K (just below melting) it is a broad Gaussian.
line at the same frequency as that of solution hemoglobin in the T state. A similar study on an R-state hemoglobin (NES-des-Arg) produced nearly the same results until the melting point, above which the clear manifestation of R/T distinguishability became evident. Some studies were also conducted on the model compound—protoporphyrin IX complexed with 3-methylimidazole. At room-temperature solution conditions, its Fe-His stretching frequency is a symmetric broad line (~30 cm⁻¹) located at 220 cm⁻¹ (16–19). Upon lowering the temperature to 77 K it narrows and shifts to 230 cm⁻¹ like the hemoglobins.

**DISCUSSION**

Only two of the strong lines in the hemoglobin Raman spectra have Gaussian lineshapes under solution conditions. Upon lowering the temperature to 10 K, the line at ~300 cm⁻¹ remains broad but some structure becomes apparent. We conclude that at least part of the broadening for this mode results from the trivial mechanism of a near coincidence in frequency of two or more different normal modes. It is only the Fe-His mode that displays a significant temperature dependence in both its linewidth and frequency (plotted in Fig. 4). This dependence can be separated into two qualitatively different phenomena—a phase transition at the ice-freezing point (20) and a continuous temperature dependence below freezing. We explore the latter effect first.

The behavior of the linewidth and frequency of the Fe-His mode shown on the right side of Fig. 4 is consistent with a process that has an Arrhenius temperature dependence—i.e., \( f = f_0 + f e^{-\Delta E/kT} \), in which the variable \( f \) has a limiting value \( f_0 \). Reorientational broadening of Raman lines can be understood by such a process (21). The mean squared displacement \( \langle x^2 \rangle \) of atomic positions also has this activation energy behavior because it depends on the population of excited vibrational levels. Such processes have been shown to be important in the understanding of the temperature dependence of x-ray scattering from protein crystals (1, 22). In studies of myoglobin crystals, Frauenfelder et al. (1, 22), by comparing the x-ray and Mössbauer results, have been able to determine \( \langle x^2 \rangle \) resulting from the sum of contributions from conformational substates, \( \langle x^2 \rangle_o \), and from the vibrational mean squared displacement, \( \langle x^2 \rangle_v \). These two contributions can be distinguished by their temperature dependence. \( \langle x^2 \rangle_v \) is expected to “freeze out” at a higher level of disorder due to a locking in of substates as the thermal noise level drops below the energetic barriers separating those substates. Vibrational disorder, \( \langle x^2 \rangle_v \), on the other hand, should decrease smoothly to the level inherent in the vibrational zero-point energy.

The temperature dependence of the linewidth of the Fe-His mode displays a behavior consistent with it arising from \( \langle x^2 \rangle_v \). As the temperature is lowered the linewidth becomes Lorentzian and smoothly decreases to 8 cm⁻¹, a width comparable to the narrowest of the homogeneously broadened porphyrin modes. There is no indication of significant broadening due to conformational substates. An extreme example of conformational substate broadening, such as chain heterogeneity, would result in the resolution of two separate peaks. Although this has been shown to account for the lineshape under solution conditions (16), it is clearly not the case in frozen hemoglobin. No evidence of multiple peaks was seen in either slow or fast frozen samples. On the other hand, some of the broadening in the line at ~300 cm⁻¹ could have contributions from conformational substates.

To account for the temperature dependence of the linewidth we first consider vibrational hot bands (transitions originating from \( \nu = 0 \)) of the Fe-His stretching mode. This and other broadening mechanisms that consider only a single vibrational potential predict that as the temperature is increased perturbations to the harmonic potential result in asymmetric broadening to the low frequency side of the line, possibly displaying submaxima but with the 0–1 transition always being the most prominent. The data (Fig. 3) clearly show a nearly symmetric Fe-His mode at all temperatures below freezing and a large (13 cm⁻¹) shift in its peak frequency as a function of temperature. Another possible temperature-activated broadening mechanism is vibrational dephasing resulting from anharmonically coupled modes (23–25). This process predicts a temperature-dependent linewidth and center frequency that are consistent with our observed data.

Dephasing gives rise to a broadening and shifting of a given vibrational mode due to anharmonic coupling with another mode which, as it becomes excited and relaxed through thermal fluctuations, modulates the frequency and width of the observed mode. For a simple four-level system the temperature dependence of the observed Raman frequency, \( \nu \), and linewidth, \( \Gamma \), may be given by (24, 25)

\[
\frac{\omega_0 - \omega}{\omega_0} = A e^{-E_{\text{tot}}}
\]

and

\[
\frac{\Gamma - \Gamma_0}{\Gamma_0} = B e^{-E_{\text{tot}}}
\]

Here \( \omega_0 \) is the uncoupled (low temperature limit) Raman frequency of width \( \Gamma_0 \) and \( E_{\text{tot}} \) is the quantum energy of the coupling mode. \( A \) and \( B \) are constants that contain the anharmonic coupling frequency shift \( \delta\omega \) and the lifetime of the coupling mode (\( \tau \)).

The predictions of this dephasing model (24, 25) are consistent with our data on the Fe-His stretching mode. Within experimental error both the frequency and the linewidth have the same temperature dependence. The plot of \( -\ln(\Delta\omega/\omega_0) \) or \( [\ln(\Delta\Gamma/\Gamma_0)] \) versus \( 1/T \), displayed in Fig. 4, is a straight line with a slope that yields an activation energy of \( \approx 200 \text{ cm}^{-1} \) (2.5 \times 10⁻² eV). Furthermore, the ratio of \( \Delta\omega / \Delta\Gamma \) gives a temperature-independent value of \( \approx 1 \), the regime in which vibrational exchange is expected to occur. This constant ratio al-

![Fig. 4. Plots of the temperature dependence of the peak position of the Fe-His stretching mode as a function of temperature (right trace) and the logarithmic dependence of the peak position versus the reciprocal temperature (left trace).](image-url)
follows the parameters $\omega_0$ and $\tau$ to be extracted from the intercept of the plots. Values of $\omega_0 \approx -70 \text{ cm}^{-1} (9 \times 10^{-3} \text{eV})$ and $\tau \approx 0.1 \text{ psec}$ are obtained. These are indicative of a highly coupled system.

The observation that the model systems of 2-methylimidazole protoporphyrin IX display qualitatively similar broadening and shifting as a function of temperature suggests that those characteristics are implicit in the heme-imidazole system and that protein vibrations do not play a major role in the dephasing phenomena below freezing. Thus the data indicate that the $\approx 200 \text{ cm}^{-1}$ mode (or set of modes yielding a $\approx 200 \text{ cm}^{-1}$ activation energy) that couples to the Fe-His stretching frequency and gives rise to the vibrational disorder is either an internal mode of the imidazole or porphyrin moiety or a librational motion of the imidazole relative to the porphyrin.

The fact that the model compound and hemoglobin have the same frequency at the low temperature limit is surprising. The assignment of this mode from isotopic substitution studies (18, 19) is consistent with a motion involving the iron and the imidazole group rather than just the iron and the nitrogen. For this to be the case, the low temperature results require an exact cancellation between the mass of the methyl group in the model compound and the effective mass from the bonding of the histidine to the polypeptide chain in the protein. Although such a coincidence is possible, other possibilities (e.g., that the $220-215 \text{ cm}^{-1}$ mode is a porphyrin mode that couples to a $200 \text{ cm}^{-1}$ Fe-His mode at room temperature) are currently being explored.

The changes in the HbA spectra induced by freezing are more complex than the low temperature behavior, although the width and position of the line indicate that the dephasing seen below freezing is still operative under solution conditions. We may interpret these results by an extension of the low temperature behavior. The R/T differences may originate from differences in the coupling of protein dynamic processes (possibly at the subunit interface) to the heme. Conformational constraints introduced by the freezing process could result in the reduction of the influence of protein degrees of freedom (for example, movement of the F-helix) upon the heme-imidazole system. It is noteworthy that whereas in phosphate buffer the R/T differences were absent in the solid state and present only in solution, spectra of solutions of HbA in 25% glycerin did not display any significant changes at the melting-freezing transition. Instead, they retained their solution spectra in the solidified state (unpublished results). These solvent effects appear to preclude a simple increase in viscosity upon freezing as the origin of the proposed conformational constraints. Instead an alteration in interactions between the protein tetramers due to dehydration, aggregation, or microcrystallization are possible explanations for the changes at the phase transition in the phosphate-buffered systems.

The presence of low frequency modes that might be able to couple to the Fe-His stretching mode in solution samples of T-state hemoglobin is suggested by the low-frequency Rayleigh wing in the Raman data where the scattering intensity for hemoglobin in the T structure is greater than that in the R structure. Although our data indicate that the low frequency wing is resonantly enhanced, these motions—apparently associated with the heme—may be coupled to the low frequency motions of the polypeptide cage surrounding the chromophore. Indeed, low-frequency protein motions in this energy range have been detected (7–10). Such low frequency modes could cause additional dephasing of the Fe-His stretching mode in the T structure and contribute to the R/T differences exhibited by this mode.

The use of Raman lineshape analysis and its temperature dependence can serve as a valuable probe of protein dynamics. Its application to hemoglobins illustrates that the observation of dynamic effects in the chromophore vibrations is dependent on the characteristics of the individual modes in question. Only those vibrations that involve localized bonds connecting independent molecular groups or manifest significant anharmonicity (or both) should display dynamic broadening. Thus, it is not surprising that the in-plane porphyrin modes of hemoglobin show very little inhomogeneous broadening. Alternatively, the Fe-His bond that is trans to the $O_2$ binding of the protein is subject to extensive coupling to other vibrational modes even in low temperature samples where the heme-imidazole system appears to be decoupled from the protein. These vibrational interactions can induce changes in frequency and width exceeding those observed as a function of changes in the quaternary structure. Thus, contributions from dynamic interactions must be considered when assessing the energetics at the active site of heme proteins and their functional implications.

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