Experimental Observation of Anharmonic Coupling of the Heme-Doming and Iron—Ligand Out-of-Plane Vibrational Modes Confirmed by Density Functional Theory

Stefan Franzen,*,† Klaus Fritsch,‡ and Scott Brewer†

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, and
Los Alamos National Laboratory, Los Alamos, New Mexico 87545.

Received: May 15, 2002; In Final Form: August 7, 2002

In the deoxy ferrous state of histidine-ligated heme proteins, the iron—histidine band ($\nu_{\text{Fe-His}}$) has been assigned as a stretching mode that involves a two-body motion involving the iron and histidine combined with a minor amount of heme doming. An analogous Raman band, $\nu_{\text{Fe-L}}$, has been observed in the proximal cavity mutant of H93G myoglobin where the Raman band of a series of nonnative axial ligands, L, can be compared. The H93G mutant of myoglobin consists of substitution of the proximal histidine, H93, by glycine. This replacement abolishes the sole covalent connection between the globin and the heme iron and creates a cavity that can be occupied by exogenous ligands, L, by dialysis. In the present study, the iron—axial-ligand out-of-plane vibration, $\nu_{\text{Fe-L}}$, for a series deoxy ferrous heme-iron adducts H93G(L) has been measured as a function of temperature, where L = imidazole (Im), 4-methyl imidazole (4-Me Im), 2-methyl imidazole (2-Me Im), 1-methyl imidazole (1-Me Im), 4-bromo imidazole (4-Br Im), and 2,4-dimethyl imidazole (2,4-diMe Im). Density functional theory calculations show that anharmonic coupling between low wavenumber heme-doming and iron—ligand out-of-plane modes can account for the calculated $\nu_{\text{Fe-L}}$, frequency shifts. These calculations parallel the experimental observed trends for temperature-dependent Raman spectra for all of the adducts except H93G-(1-Me Im).

Introduction

Iron serves as the binding site for oxygen in globins and the active site for O—O bond scission in peroxidases and oxidases. A histidine is the sole covalent connection between the protein and the heme iron active site of peroxidases, oxidases, and globins. Resonant Raman spectra of heme proteins have shown that a band in the region 200—250 cm$^{-1}$ that involves motion of both the iron and histidine is also sensitive to protein conformation and pH. The frequency of the iron—histidine mode, $\nu_{\text{Fe-His}}$, is correlated with binding strength in hemoglobin, reactivity in peroxide, and the state of ligation of the heme in cytochrome c oxidase. The frequency differences in $\nu_{\text{Fe-His}}$ suggest that ligand—iron bonding is important for differences in reactivity among these heme proteins. However, comparisons among different proteins are based on the assumption that the vibrational mode is similar in nature in different proteins. If large differences in conformational coupling of the iron—histidine anharmonic coupling of this mode to other normal modes in the protein exist, then the significance of the frequency comparisons do not reflect differences in iron—histidine bonding alone.

The higher frequency of the iron—histidine vibrational mode in peroxidases ($\nu_{\text{Fe-His}} \approx 243—245$ cm$^{-1}$), when compared to globins, has been understood in terms of the stronger hydrogen bond between an aspartate and the imidazole N—H as opposed to the weak H bond between serine and the imidazole N—H in the globins. The proposed correlation with the frequency of the $\nu_{\text{Fe-His}}$ mode suggests that the higher the frequency of this mode, the greater the electron density on the heme iron and, therefore, the greater the extent of activation of bound oxygen.

On the other hand, the lower frequency of $\nu_{\text{Fe-His}}$ observed in cytochrome c oxidase may be due to weaker hydrogen bonding inferred from X-ray crystal data. The functional importance of the lower frequency in cytochrome c oxidase is not well understood. The enzyme dehaloperoxidase from Amphitrite Ornata that has $\nu_{\text{Fe-His}} \approx 232$ cm$^{-1}$ intermediate between a globin and a peroxidase has a structure that is consistent with a globin structure, but this frequency is suggestive of an ability to activate bound oxygen. Guanylyl cyclase has a still lower frequency of $\nu_{\text{Fe-His}} \approx 206$ cm$^{-1}$ indicative of a labile histidine as required by the function of the protein in signal transduction. The origin of the frequency differences in these proteins appears to depend on the strength of the iron—histidine bonding rather than conformational coupling suggested for hemoglobin. However, the role played by strain is not known, and the interpretation of the bond strength—frequency correlation will depend on the magnitude of anharmonic coupling that is manifested in the temperature dependence of the $\nu_{\text{Fe-His}}$ Raman band.

Frequency shifts of the $\nu_{\text{Fe-His}}$ vibrational mode have been observed as a function of temperature in the deoxy state and as a function of time following ligand photolysis. Hemoglobin provides an example of time-dependent shifts in the frequency of $\nu_{\text{Fe-His}}$. The difference in frequency between the R (high affinity) and T (low affinity) states in hemoglobin is characterized by a difference in the frequency of the $\nu_{\text{Fe-His}}$ Raman mode with $\nu_{\text{Fe-His}} \approx 224$ cm$^{-1}$ in the R state (10 ns photoproduct) and $\nu_{\text{Fe-His}} \approx 216$ cm$^{-1}$ in the T state (equilibrium). The frequency lowering has been interpreted in terms of a weakening of the iron—histidine bond in the T state relative to the R state. The frequency, $\nu_{\text{Fe-His}}$ in hemoglobin and myoglobin is also temperature dependent. Deoxy myoglobin exhibits an increase from $\nu_{\text{Fe-His}} \approx 217$ cm$^{-1}$ at ambient temperature to $\nu_{\text{Fe-His}} \approx 226$ cm$^{-1}$ at 10 K (i.e., $\approx 3$ cm$^{-1}$/100 K). The increase in band frequency of $\nu_{\text{Fe-His}}$ in deoxy hemoglobin...
shows an increase from $v_{Fe-His} \approx 216$ cm$^{-1}$ at ambient

temperature to $v_{Fe-His} \approx 224$ cm$^{-1}$ at 10 K ($\approx 3$ cm$^{-1}$/100 K).\(^6\)

Temperature-dependent shifts in the frequency of a vibrational

mode have been ascribed to anharmonicity.\(^7\) The comparison

of frequency shifts due to conformation (time-dependence) and

anharmonic coupling (temperature dependence) is considered

in this study to ascertain whether these two observations are

coupled.

The proximal cavity mutant of myoglobin H93G provides

an excellent test of the effect of bonding on different axial

ligands within the same protein. In this study, we compare a

series of iron–axial-ligand adducts in the H93G mutant of

myoglobin. The H93G mutant of myoglobin consists of

substitution of the proximal histidine, H93, by glycine.\(^8\)\(^9\) This

replacement abolishes the sole covalent connection between the

globin and the heme iron and creates a cavity that can be

occupied by exogenous ligands by dialysis. The heme-iron

adducts are analogous to heme model systems;\(^10\)\(^11\) however, the

adducts are surrounded by the globin. In the present study, the Raman spectra of
deoxy ferrous heme-iron adducts of a series of substituted imidazoles demonstrates a temperature

dependence of the $v_{Fe-L}$ Raman band, where L is the axial

ligand. Analysis within the harmonic approximation reveals that

the axial ligand out-of-plane mode, $v_{Fe-L}$, is an iron–axial-
ligand stretching vibration (50–70% of the potential energy
distribution or PED) with a minor contribution of the heme

domino coordinate (50–30% of the PED).\(^2\)\(^3\) The temperature-
dependent resonant Raman data are compared with density

functional theory (DFT) calculations. Although single mode or

intrinsic anharmonicity does not play an important role in the
temperature dependence, anharmonic multimode coupling be-
tween iron–axial out-of-plane motion (heme-domino) and the

iron-axial out-of-plane normal mode can account for the

temperature-dependent shifts observed experimentally. The

anharmonic-coupling hypothesis\(^17\) is tested and confirmed in

the present study.

### Experimental Section

Samples of H93G myoglobin with different ligands were

prepared by dialysis as discussed previously.\(^19\) The samples were

prepared in 50% glycerol/phosphate buffer mixtures (w/v). The
deoxy form of myoglobin was prepared by dialution into

deoxygenated buffer followed by reduction with excess dithion-
ite. Solutions were placed in a NMR tube spinning cell at

ambient temperature and on a gold-plated copper coldfinger

derived coordinate ($\approx 50$ Å$^2$ distribution or PED) with a minor contribution of the heme

ligand. Analysis within the harmonic approximation reveals that

the axial ligand out-of-plane mode, $v_{Fe-L}$, is an iron–axial-
ligand stretching vibration (50–70% of the potential energy
distribution or PED) with a minor contribution of the heme

domino coordinate (50–30% of the PED).\(^2\)\(^3\) The temperature-
dependent resonant Raman data are compared with density

functional theory (DFT) calculations. Although single mode or

intrinsic anharmonicity does not play an important role in the
temperature dependence, anharmonic multimode coupling be-
tween iron–axial out-of-plane motion (heme-domino) and the

iron–axial out-of-plane normal mode can account for the

temperature-dependent shifts observed experimentally. The

anharmonic-coupling hypothesis\(^17\) is tested and confirmed in

the present study.

### Density Functional Theory Calculations

The density functional theory (DFT) calculations were

performed using the MSI (Molecular Simulations, Inc.) ab initio

quantum chemical software program Dmol3.\(^24\) Dmol3 was used for

green for geometry optimization, single-point energy, and frequency

calculations of the substituted imidazole iron–porphine models
designated Por(L), where Por and L represent the porphine and

axial ligand, respectively. Comparisons of calculations of

porphine–CO models with experimental results have been

discussed elsewhere.\(^25\)\(^26\) These calculations used the DNP basis

set and the GGA functional in the gas phase. The graphical

user interface InsightII (Accelrys, San Diego, CA) was used to

build the models and to visualize the eigenvector projections

calculated by Dmol3. The DFT calculations were performed at

the North Carolina Supercomputing Center (NCSC) on the IBM

RS/6000 SP. Normal coordinate analysis was performed using

FCART01\(^27\) to calculate the potential energy distributions (PED)

for the normal modes. The intrinsic mode anharmonicities were
determined by calculating potential energy surfaces (PES) along the

iron-axial out-of-plane normal mode formed by calculating the
energy at displacements along the eigenvector from the

optimized geometry. A fourth-order polynomial ($E(Q) = a_0 + a_1Q + a_2Q^2 + a_3Q^3 + a_4Q^4$) was used to fit the PES and these

parameters were used in the

Numerov–Cooley\(^28\)\(^29\) method to calculate the energies and wave functions for the first five vibrational levels for this normal mode. The intrinsic mode

anharmonicity was then calculated from the average energy

($cm^{-1}$) spacing difference between the first five adjacent vibrational levels (i.e., difference between $v_1 - v_2$, $v_1 - v_3$, $v_1 - v_4$, $v_1 - v_5$). Anharmonic multimode coupling was investi-
gated by projecting along the eigenvector of normal modes

containing iron–axial out-of-plane motions (heme-domino) and
calculating the shift in frequency in the iron–axial out-of-
plane normal mode relative to the equilibrium calculated

frequency. The calculated shifts of the substituted imidazoles

were normalized to imidazole by

$$\Delta \nu_{norm} = \frac{PED_{Fe-N}(Im)}{PED_{Fe-N}(L)} \times \frac{MW(Im)}{MW(L)}$$

where $\Delta \nu_{norm}$ is the normalized shift in the iron-axial out-of-
plane mode, PED is the potential energy distribution (% Fe–
Ne stretching) of this mode, and MW is the molecular weight

of imidazole $(Im)$ or the substituted imidazole ligands $(L)$ to

account for the varying molecular weights and PED for the

substituted imidazole ligands.

### Results

The temperature dependence of the Raman spectra of H93G-

(Im) and H93G(2-Me Im) are shown in Figures 1 and 2,

respectively. A complete analysis of the spectra was carried out

by fitting to multiple Gaussian bands to determine which bands

shift as a function of temperature (see the Supporting Informa-
tion). This analysis is essential because one interpretation of

the multiple bands in the region from 190 to 240 cm$^{-1}$ is

that there are several conformations of the protein present.\(^14\)\(^30\)

However, a fit of this region to three to five independent

Gaussians (depending on the ligand) yielded significant shifts

from a single Gaussian band or a pair of Gaussians. The

weighted mean frequency of these Gaussians showed a consis-
tent trend to higher frequency and an increase in asymmetry as

the temperature was lowered consistent with the anharmonic

coupling model\(^17\) (see the Supporting Information). To higher

energy, the peaks at $\approx 263$ and $\approx 283$ cm$^{-1}$ present in all of the

Raman spectra do not show a consistent temperature-dependent

shift. A nonshifting peak at $\approx 250$ cm$^{-1}$ appears in spectra of

all adducts except 4-Br imidazole (data not shown). There is a

nonshifting mode at $\approx 240$ cm$^{-1}$ in all of the adducts except
H93G (2-Me Im). This band at 240 cm⁻¹ is seen very clearly in both the horse heart and sperm whale myoglobin resonance Raman spectra and has been assigned as 9.31. In each of the adducts of H93G myoglobin, there was a single Raman band that increased in frequency as the temperature was lowered from 293 to 83 K. The frequency shift for the temperature-dependent Fe-L band for each of six adducts is reported in Table 1 along with that of wild-type horse heart myoglobin.

The H93G mutant can be prepared with no exogenous ligand present; however, the iron in this species is five coordinate with H₂O as the fifth ligand. 32 The Raman spectrum seen in Figure 3 has no iron-ligand out-of-plane mode in the region of the spectrum from 180 to 240 cm⁻¹. There is one pronounced (nonshifting) peak in H93G(H₂O) at ≈236 cm⁻¹ assigned as ν₉. The H93G(H₂O) species serves as a consistency test for the observation that only the band that corresponds to Fe-L shifts with temperature. The vibrational modes seen in the spectrum of Figure 3 are nearly the same as the nonshifting modes seen in the spectra of the other H93G(L) ligand adducts. Consistent with the results for the imidazole adducts H93G(L), none of the bands in H93G(H₂O) show any temperature dependence.

The shift in the peak frequency of the iron-ligand out-of-plane mode for all of the ligands is plotted as a function of temperature in Figure 4. The peak shift was determined based on fits of all of the data to 3-5 Gaussians over the spectral range 190-240 cm⁻¹. The lines through the data points were obtained by a linear least-squares fit. The slopes and room-temperature values of the frequencies for each ligand are given in Table 1. Comparing the slopes given in Table 1 shows that Fe-L for H93G(2-Me Im) has a temperature dependence more than twice as large as that of either wild-type horse heart myoglobin or H93G(4-Me Im). The H93G(1-Me Im) adduct studied consisted of the di-15 N isotopomer. The isotopomer of 1-Me Im was used because the Raman spectrum for H93G(1-Me Im) shows a Fermi resonance that would obscure the frequency shifts reported. 23 H93G(1-Me Im) also shows a frequency shift twice as large as that of H93G(4-Me Im). This result is surprising because the structures of 4-Me Im and 1-Me Im are quite similar in the proximal pocket.

**Table 1:** Iron–Ligand Out-of-Plane Mode Frequency as a Function of Temperature for Axial Ligand Adducts to Deoxy Ferrous H93G Myoglobin

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Slope/100 K</th>
<th>100 K (cm⁻¹)</th>
<th>293 K (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Me Im</td>
<td>2.5 ± 0.3</td>
<td>223.2</td>
<td>218.7</td>
</tr>
<tr>
<td>Horse heart</td>
<td>3.0 ± 0.2</td>
<td>223.0</td>
<td>217.7</td>
</tr>
<tr>
<td>1-Me Im</td>
<td>4.6 ± 0.2</td>
<td>238.6</td>
<td>229.2</td>
</tr>
<tr>
<td>Imidazole</td>
<td>5.5 ± 0.3</td>
<td>230.6</td>
<td>220.6</td>
</tr>
<tr>
<td>4-Br-Im</td>
<td>5.9 ± 0.4</td>
<td>206.3</td>
<td>194.2</td>
</tr>
<tr>
<td>2,4-diMe Im</td>
<td>6.1 ± 0.4</td>
<td>206.7</td>
<td>194.5</td>
</tr>
<tr>
<td>2-Me Im</td>
<td>6.7 ± 0.5</td>
<td>221.0</td>
<td>207.1</td>
</tr>
</tbody>
</table>

H93G(2-Me Im). This band at ≈240 cm⁻¹ is seen very clearly in both the horse heart and sperm whale myoglobin resonance Raman spectra and has been assigned as ν₉. In each of the adducts of H93G myoglobin, there was a single Raman band that increased in frequency as the temperature was lowered from 293 to 83 K. The frequency shift for the temperature-dependent Fe-L band for each of six adducts is reported in Table 1 along with that of wild-type horse heart myoglobin.

The H93G mutant can be prepared with no exogenous ligand present; however, the iron in this species is five coordinate with H₂O as the fifth ligand. The Raman spectrum seen in Figure 3 has no iron-ligand out-of-plane mode in the region of the spectrum from 180 to 240 cm⁻¹. There is one pronounced (nonshifting) peak in H93G(H₂O) at ≈236 cm⁻¹ assigned as ν₉. The H93G(H₂O) species serves as a consistency test for the observation that only the band that corresponds to Fe-L shifts with temperature. The vibrational modes seen in the spectrum of Figure 3 are nearly the same as the nonshifting modes seen in the spectra of the other H93G(L) ligand adducts. Consistent with the results for the imidazole adducts H93G(L), none of the bands in H93G(H₂O) show any temperature dependence.

The shift in the peak frequency of the iron-ligand out-of-plane mode for all of the ligands is plotted as a function of temperature in Figure 4. The peak shift was determined based on fits of all of the data to 3-5 Gaussians over the spectral range 190-240 cm⁻¹. The lines through the data points were obtained by a linear least-squares fit. The slopes and room-temperature values of the frequencies for each ligand are given in Table 1. Comparing the slopes given in Table 1 shows that Fe-L for H93G(2-Me Im) has a temperature dependence more than twice as large as that of either wild-type horse heart myoglobin or H93G(4-Me Im). The H93G(1-Me Im) adduct studied consisted of the di-15 N isotopomer. The isotopomer of 1-Me Im was used because the Raman spectrum for H93G(1-Me Im) shows a Fermi resonance that would obscure the frequency shifts reported. H93G(1-Me Im) also shows a frequency shift twice as large as that of H93G(4-Me Im). This result is surprising because the structures of 4-Me Im and 1-Me Im are quite similar in the proximal pocket.

**Table 1:** Iron–Ligand Out-of-Plane Mode Frequency as a Function of Temperature for Axial Ligand Adducts to Deoxy Ferrous H93G Myoglobin

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Slope/100 K</th>
<th>100 K (cm⁻¹)</th>
<th>293 K (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Me Im</td>
<td>2.5 ± 0.3</td>
<td>223.2</td>
<td>218.7</td>
</tr>
<tr>
<td>Horse heart</td>
<td>3.0 ± 0.2</td>
<td>223.0</td>
<td>217.7</td>
</tr>
<tr>
<td>1-Me Im</td>
<td>4.6 ± 0.2</td>
<td>238.6</td>
<td>229.2</td>
</tr>
<tr>
<td>Imidazole</td>
<td>5.5 ± 0.3</td>
<td>230.6</td>
<td>220.6</td>
</tr>
<tr>
<td>4-Br-Im</td>
<td>5.9 ± 0.4</td>
<td>206.3</td>
<td>194.2</td>
</tr>
<tr>
<td>2,4-diMe Im</td>
<td>6.1 ± 0.4</td>
<td>206.7</td>
<td>194.5</td>
</tr>
<tr>
<td>2-Me Im</td>
<td>6.7 ± 0.5</td>
<td>221.0</td>
<td>207.1</td>
</tr>
</tbody>
</table>

H93G(2-Me Im). This band at ≈240 cm⁻¹ is seen very clearly in both the horse heart and sperm whale myoglobin resonance Raman spectra and has been assigned as ν₉. In each of the adducts of H93G myoglobin, there was a single Raman band that increased in frequency as the temperature was lowered from 293 to 83 K. The frequency shift for the temperature-dependent Fe-L band for each of six adducts is reported in Table 1 along with that of wild-type horse heart myoglobin.

The H93G mutant can be prepared with no exogenous ligand present; however, the iron in this species is five coordinate with H₂O as the fifth ligand. The Raman spectrum seen in Figure 3 has no iron-ligand out-of-plane mode in the region of the spectrum from 180 to 240 cm⁻¹. There is one pronounced (nonshifting) peak in H93G(H₂O) at ≈236 cm⁻¹ assigned as ν₉. The H93G(H₂O) species serves as a consistency test for the observation that only the band that corresponds to Fe-L shifts with temperature. The vibrational modes seen in the spectrum of Figure 3 are nearly the same as the nonshifting modes seen in the spectra of the other H93G(L) ligand adducts. Consistent with the results for the imidazole adducts H93G(L), none of the bands in H93G(H₂O) show any temperature dependence.

The shift in the peak frequency of the iron-ligand out-of-plane mode for all of the ligands is plotted as a function of temperature in Figure 4. The peak shift was determined based on fits of all of the data to 3-5 Gaussians over the spectral range 190-240 cm⁻¹. The lines through the data points were obtained by a linear least-squares fit. The slopes and room-temperature values of the frequencies for each ligand are given in Table 1. Comparing the slopes given in Table 1 shows that Fe-L for H93G(2-Me Im) has a temperature dependence more than twice as large as that of either wild-type horse heart myoglobin or H93G(4-Me Im). The H93G(1-Me Im) adduct studied consisted of the di-15 N isotopomer. The isotopomer of 1-Me Im was used because the Raman spectrum for H93G(1-Me Im) shows a Fermi resonance that would obscure the frequency shifts reported. H93G(1-Me Im) also shows a frequency shift twice as large as that of H93G(4-Me Im). This result is surprising because the structures of 4-Me Im and 1-Me Im are quite similar in the proximal pocket.
TABLE 2: Iron-Ligand Out-of-Plane Mode DFT Calculated Frequencies, Potential Energy Distributions, and Intrinsic Mode Anharmonicities

<table>
<thead>
<tr>
<th>adduct</th>
<th>Fe–Nc distance (Å)</th>
<th>Fe–Nc binding energy (kJ/mol)</th>
<th>(v_{Fe-L} (cm^{-1}))</th>
<th>PED</th>
<th>[(\Delta)] single mode (cm(^{-1}))</th>
<th>doming mode (cm(^{-1}))</th>
<th>[(\Delta)] multimode (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Me Im</td>
<td>2.19</td>
<td>75.2</td>
<td>143.2</td>
<td>44%</td>
<td>(+)0.3</td>
<td>76.8</td>
<td>0.4</td>
</tr>
<tr>
<td>1-Me Im</td>
<td>2.16</td>
<td>75.3</td>
<td>160.5</td>
<td>73%</td>
<td>(−)0.3</td>
<td>80.9</td>
<td>5.1</td>
</tr>
<tr>
<td>imidazole</td>
<td>2.18</td>
<td>74.0</td>
<td>169.9</td>
<td>66%</td>
<td>(−)0.2</td>
<td>87.3</td>
<td>1.0</td>
</tr>
<tr>
<td>4-Br Im</td>
<td>2.19</td>
<td>70.4</td>
<td>142.9</td>
<td>64%</td>
<td>(+)0.1</td>
<td>70.3</td>
<td>3.3</td>
</tr>
<tr>
<td>2,4-diMe Im</td>
<td>2.20</td>
<td>78.6</td>
<td>155.5</td>
<td>55%</td>
<td>(+)3.2</td>
<td>84.2</td>
<td>3.7</td>
</tr>
<tr>
<td>2-Me Im</td>
<td>2.21</td>
<td>74.8</td>
<td>163.0</td>
<td>61%</td>
<td>0.0</td>
<td>74.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>

\(^a\) Binding energy = (Fe–Nc)\(_{\text{eq}}\) – (Fe–Nc)\(_{\text{equilibrium distance}}\)

\(^b\) Average anharmonicity over first five vibrational levels. (−) Decrease in energy spacing; (+) increase in energy spacing going up in vibrational levels.

\(^c\) Energy of the low wavenumber heme-doming normal mode that was displaced by 0.5 normal coordinate units along its eigenvector projection.

\(^d\) Normalized shift (\(\Delta\)) in the iron–ligand out-of-plane mode of the substituted imidazole ligands to imidazole to account for the different molecular weights and % Fe–N stretching of the iron–ligand out-of-plane mode as shown above.

Figure 5. DFT calculated eigenvector projections of the iron–ligand out-of-plane mode, \(v_{Fe-L}\), and the heme-doming mode for the porphine models. 4-Me Im (A), 1-Me Im (B), imidazole (C), 4-Br Im (D), 2,4-diMe Im (E), and 2-Me Im (F).

The vibrational spectrum of each adduct within the harmonic approximation was obtained by finite difference based on the energies of a DFT calculation for a model complex that consisted of an iron porphine with an axial ligand. Table 2 gives the calculated equilibrium distances, binding energies, and frequencies of the Fe–Nc bond for the various substituted porphine models with axial imidazole ligands. The mode identified as \(v_{Fe-L}\) from the calculation, i.e., the mode that shows the greatest PED for the Fe–Nc stretching coordinate, is found in the wavenumber range from 130 to 160 cm\(^{-1}\) for all model adducts as shown in Table 2. Consistent with a previous analysis, 23 the Fe–Nc stretching component of the iron–ligand out-of-plane mode is between 44 and 73% for the porphine adducts. However, the calculated frequencies for \(v_{Fe-L}\) are on average \(\approx 27\%\) lower than the corresponding observed Raman frequencies given in Table 1. This difference is somewhat surprising because DFT calculations of high-frequency modes in metalloporphines tend to be much more accurate. 25,26,34,35 The mode calculated in the 228–239 cm\(^{-1}\) mode, i.e., closest to the experimentally observed frequency, is predominantly an iron out-of-plane motion that has little Fe–Nc stretching.

In addition to the contribution of the internal coordinates to the vibrational modes within the harmonic approximation, anharmonic coupling of a low-frequency heme-doming mode with the iron–ligand out-of-plane mode has been proposed based on a two-mode quantum mechanical model. 17 The eigenvectors obtained from DFT calculations shown in Figure 5 correspond to a heme-doming mode that ranges from 70 to 90 cm\(^{-1}\) in the various adducts as given in Table 2. The value of 70–90 cm\(^{-1}\) calculated here for the iron-doming mode is in the range of 50–100 cm\(^{-1}\) found for previous calculations 41,42 and experiments. 43 On the basis of the PED, we have performed the analysis of anharmonicity using the 130–160 cm\(^{-1}\) as the model for the \(v_{Fe-L}\) mode including both the intrinsic anharmonicity and anharmonic coupling to the low wavenumber heme-doming mode. The intrinsic anharmonicity of the iron–ligand mode was calculated using potential energy surfaces derived from projections along the eigenvectors shown in Figure 5. The corresponding PES of the iron–ligand mode for each of the substituted imidazoloporphine models with the first five vibrational wave functions calculated using the Numerov–Cooley method is shown in the Supporting Information.
Experimental Observation of Anharmonic Coupling

Figure 6. Plot of the iron–ligand out-of-plane frequency temperature dependence versus the DFT calculated normalized intermediate anharmonic coupling between the low-frequency heme-doming mode and the iron–ligand out-of-plane mode for the substituted imidazole ligands.

Anharmonic multimode coupling of the heme-doming and iron–ligand out-of-plane mode was studied by displacing along the normal mode (eigenvector) of low wavenumber heme-doming mode and calculating the resulting shift in the calculated frequency of the $v_{Fe-L}$ mode. The heme-doming modes (Figure 5) were calculated to be 70–90 cm$^{-1}$ and were displaced by 0.5 mass-weighted normal coordinate units to calculate the relative magnitude of anharmonic multimode coupling for the various imidazole ligands. Table 2 shows the calculated shifts that were normalized (eq 1) to take into account the different masses and percentages of Fe–Nε stretching motion in the iron–ligand out-of-plane mode for the substituted imidazole ligands (see also THE Supporting Information). Figure 6 shows the linear dependence of the slope of the experimental Raman temperature dependence of the iron–ligand out-of-plane mode (Table 1) and the DFT calculated normalized intermediate anharmonic coupling between the low-frequency heme-doming mode and $v_{Fe-L}$ (Table 2). This plot illustrates that the calculated normalized shifts parallel the experimental data, with the exception of the model porphine adduct of 1-Me Im. The ordering of the magnitude of the shifts is Por(2,4-diMe Im) > Por(2,4-diMe Im) > Por(Im) > Por(4-Br Im) in agreement with experiment as seen by comparing Tables 1 and 2. Although the Im ligand lies below the correlation line in Figure 6, it follows the ordering of anharmonicities. The magnitude of the calculated shift is arbitrary in the current model. It is dependent on the magnitude of the displacement in the anharmonically coupled heme-doming mode. To obtain a quantitative comparison, displacement along a number of coordinates must be carried out combined with vibrational frequency calculations at each displacement. The shift can be calculated as the expectation value of the frequency shift (harmonic shift approximation) as discussed elsewhere. Several other possible modes were investigated that could potentially give rise to anharmonic coupling (see the Supporting Information).

Discussion

Despite a large number of studies of the iron–histidine and iron–imidazole resonant Raman band in heme proteins, there have been relatively few studies of the temperature dependence of the $v_{Fe-His}$ vibrational mode in native heme proteins. The results for various ligands in H93G(L) suggest that the temperature dependences can be substantial. This appears to be particularly true for the iron adduct of the hindered ligand 2-methyl imidazole. The H93G(2-Me Im) adduct is interesting because it was used in the first heme models used to assign the iron–histidine stretch. The 2-methyl imidazole adduct of heme has also been interpreted as a model for T-state hemoglobin. We have recently shown that photolyzed H93G(2-Me Im) shows a large shift in the $v_{Fe-L}$ band in the 8 ns photoproduct analogous to the photolyzed R-state of HbCO. This comparison is further substantiated in the present data because both T-state hemoglobin and H93G(2-Me Im) appear to have a relatively large temperature dependence of their $v_{Fe-His}$ bands. On the other hand, the temperature dependence of $v_{Fe-L}$ in H93G(4-Me Im) is nearly identical to that of horse heart myoglobin consistent with the fact that 4-Me Im is chemically most closely related to imidazole of all of the ligands that can be substituted into H93G. It is interesting that H93G(4-Me Im) and H93G(2-Me Im) represent the smallest and largest temperature dependence, respectively.

The calculated value for the shift of $v_{Fe-L}$ of H93G(1-Me Im) due to anharmonic coupling does not follow the trend of the other ligands. The $v_{Fe-L}$ Raman band for the natural abundance isotopomer of H93G(1-Me Im) is the only one that shows a Fermi resonance. The dideutero isotopomer of 1-Me Im removes the splitting of the Fermi resonance; however, the DFT calculation corresponds to the natural abundance isotopomer. The Fermi resonance of H93G(1-Me Im) indicates that anharmonic coupling to modes other than the heme doming is likely present for 1-Me Im. Therefore, the anharmonic coupling model applied to the DFT calculation does not necessarily account for the true anharmonic coupling present in H93G(1-Me Im). The difference in anharmonic coupling is remarkable because the structures of H93G(1-Me Im) and H93G(4-Me Im) have been shown to be nearly identical by X-ray crystallography. However, 1-Me and 4-Me Im differ in their hydrogen bonding because 4-Me Im can form a strong H bond with serine 92, whereas 1-Me Im cannot. In fact, the 1-Me Im adduct is the only substituted imidazole that is incapable of hydrogen bonding. Although the link between this observation and the spectral anomalies has not yet been established, these facts do suggest that H93G(1-Me Im) should be considered separately from the other ligands.

The DFT calculations suggest that intrinsic (single-mode) anharmonicity of the iron–ligand out-of-plane mode does not explain the observed frequency shifts of this band in the temperature dependent Raman spectra. On the other hand, anharmonic coupling of a low-frequency heme-doming mode with the iron–ligand stretch (intermode coupling) does account relatively well for the data. The success of this calculated model verifies conclusions obtained previously using a two-mode quantum mechanical model. Moreover, the calculation suggests that additional effects due to off-axis tilting are small relative to the iron-doming mode contribution to anharmonic coupling.

The data obtained in the present study can be compared with 8 ns photoproduct spectra obtained on some of the same ligands. The relative magnitude of shifts in the $v_{Fe-L}$ mode is quite different in that case. The ordering of the photoproduct shifts in 75% glycerol/buffer is H93G(2-Me Im) > H93G(4-Me Im) > H93G(1-Me Im) = H93G(Im). The origin of the shifts in the photoproduct spectra does not appear to depend on the anharmonic coupling to the iron-doming mode.

The present study supports interpretations of the frequency in terms of bonding and strain that have been advanced in a range of heme proteins. The magnitude of frequency shifts in
H93G(4-Me Im) is similar to wild type myoglobin. Moreover, the success of the anharmonic coupling model strongly suggests that the apparent non-Gaussian line shape of the $\nu_{Fe-L}$ Raman band arises from the presence of Raman bands from modes that are not anharmonically shifted rather than conformational substrates of myoglobin.\textsuperscript{14,44} The A-states of myoglobin are observed in the CO-bound form as multiple infrared CO stretching bands.\textsuperscript{45-47} The B states of myoglobin are observed in the photoprod-uct, i.e., when the CO ligand is still present in the protein but not bound to the heme iron.\textsuperscript{28-30} Although it is logical to seek conformational substrates with barriers of similar height in the deoxy form, the present study suggests that the $\nu_{Fe-L}$ Raman band does not provide evidence for such conformational substrates in deoxy myoglobin.

**Conclusions**

Temperature-dependent Raman frequency shifts of the iron–ligand out-of-plane mode, $\nu_{Fe-L}$, have been observed for a variety of substituted imidazole ligands in the H93G mutant of myoglobin. The magnitude of the frequency shift was dependent on the identity of the substituted imidazole ligand present and decreased in the order H93G(2-Me Im) > H93G(2,4-diMe Im) > H93G(4-Br Im) > H93G(Im) > H93G(1-Me Im) > H93G(4-Me Im). With the exception of the H93G(1-Me Im) adduct, the trend is the same as that calculated using an anharmonic intermode coupling by DFT methods. DFT calculations support earlier suggestions that this normal mode had a significant (44–73%) component of Fe–Ne stretching motion; however, the calculated frequency of the $\nu_{Fe-L}$ mode is substantially lower than the observed mode frequency. The DFT calculations are consistent with a doming mode at lower frequency than the $\nu_{Fe-L}$ mode that is strongly anharmonically coupled to the $\nu_{Fe-L}$ mode. These observations corroborate the results of other studies both regarding the PED of the $\nu_{Fe-Hu}$ mode\textsuperscript{23,51} and the frequency of the doming\textsuperscript{11,12} as well as its anharmonic coupling.\textsuperscript{17} The success of an intermode anharmonic coupling model is a significant step both in the field of heme protein spectroscopy and in the development of DFT methods for the analysis of spectroscopy.

**Acknowledgment.** S.F. acknowledges a Director’s Fellowship at Los Alamos National Laboratory. Raman data were obtained at Los Alamos National Laboratory. S.F. acknowledges support by NSF Grant MCB-9874895. S.B. was supported by the NIH Biotechnology Training Grant T32-GM08776. We thank S. Stavrov for helpful discussions. S.F. and S.B. acknowledge support by the North Carolina Supercomputer Center (NCSC).

**Supporting Information Available:** A complete analysis of the spectra. This material is available free of charge via the Internet at http://pubs.acs.org.