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Resonance Raman Spectra of Methemoglobin Derivatives. Selective Enhancement of Axial Ligand Vibrations and Lack of an Effect of Inositol Hexaphosphate[†]

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ABSTRACT: Resonance Raman spectra have been obtained for the OH^-, N_3^-, and F^- derivatives of methemoglobin by excitation in the 550–650-nm region. A selective enhancement with excitation in the charge-transfer bands is observed for peaks at 413 and 497 cm^{-1} and a doublet at 471 and 443 cm^{-1} in the N_3^-, OH^-, and F^- complexes, respectively. These peaks are assigned to Fe–axial ligand stretches on the basis of: (1) a 20-cm^{-1} shift of the 497-cm^{-1} peak of the hydroxide complex to lower energy on isotopic substitution of $^{18}OH^-$ for $^{16}OH^-$; (2) the proximity of the 413-cm^{-1} Raman peak to the 421-cm^{-1} IR peak previously assigned to the Fe–N_3^- stretch in a model heme-azide complex [Ogoshi, H., Watenabe, E., Yoshida, Z., Kincaid, J., and Nakamoto, K. (1973), *J. Am. Chem. Soc. 95*, 2845]; (3) the selective appearance of the 471- and 443-cm^{-1} peaks in the Raman spectra of the F^- complex. The doublet observed at 471 and 443 cm^{-1} in the F^- derivative may reflect a heterogeneity in the heme cavity due to hydrogen bonding of H_2O to the F^- ligand in both the α and β subunits, as has been previously suggested based on x-ray diffraction results (Deatherage, J. F., Loe, R. S., and Moffat, K. (1976), *J. Mol. Biol. 104*, 723). It is suggested that the frequency of the Fe–F^- vibration reflects the out-of-plane distortion of the Fe from the heme plane. The lack of a shift in the frequency of the Fe–F^- vibration suggests that there is little or no movement of the iron with respect to the heme plane upon the addition of inositol hexaphosphate, which is thought to alter the allosteric equilibrium between the R and T forms of methemoglobin. This result is consistent with a recent x-ray crystallographic study of an IHP complex of MetHb-F^- (Fermi G., and Perutz, M. F. (1977), *J. Mol. Biol. 114*, 421). Excitation profile measurements suggest that the charge-transfer band in methemoglobin OH^- like that in methemoglobin N_3^- is z polarized, while in methemoglobin F^- the charge transfer transition is mixed with a π to π^* transition.

Resonance Raman spectroscopy can serve as a structural probe for biological molecules such as hemoglobin and myoglobin (Spiro, 1975, and references therein; Yamamoto et al., 1973; Kitagawa et al., 1975; Ozaki et al., 1976). Upon excitation within the absorption bands of the heme chromophore a selective enhancement occurs in the intensity of the Raman peaks resulting from heme vibrations (Spiro, 1975). In previous reports the dominant Raman bands which have been observed appear to result from in-plane porphyrin macrocyclic modes and occur at energies between 600 and 1700 cm^{-1}. This is because excitation occurred within π to π^* electronic transitions of the porphyrin macrocycle such as in the α, β, and Soret bands. The energies of some of these Raman peaks have been shown to be sensitive to the oxidation state, spin state, and/or

planarity of the metal with respect to the porphyrin plane (Spiro, 1975; Spaulding et al., 1975). However, vibrational modes of the iron in heme complexes, such as Fe–axial ligand modes, are rarely observed (Brunner, 1974; Spiro and Burke, 1976) because the π orbitals involved in the α, β, and Soret bands are poorly conjugated with the metal orbitals (Asher and Sauer, 1976). Unfortunately, these vibrational modes are precisely the ones that contain the greatest information on ligand binding in heme proteins. In addition, these modes would be expected to be sensitive to constraints imposed by the protein such as the proposed tension on the heme iron by the proximal histidine during the transition between the R and T allosteric forms (Perutz, 1972).

Recently, Asher and Sauer (1976) demonstrated the specific enhancement of vibrational modes involving the metal when excitation occurred within the charge-transfer bands of manganese(III) etioporphyrin, suggesting that a similar enhancement of vibrational modes which involve the metal should occur upon excitation into charge-transfer bands of heme. The heme in methemoglobin, like manganese(III) porphyrins, has electronic transitions between 600 and 640 nm which have been assigned to charge-transfer bands (see Smith and Williams, 1970). Excitation within these bands should enhance vibrational modes such as axial ligand stretches. We have utilized a tunable dye laser which can excite within these charge-transfer bands and report here a study of ligation properties of methemoglobin (Asher et al., 1977; Asher, 1976).
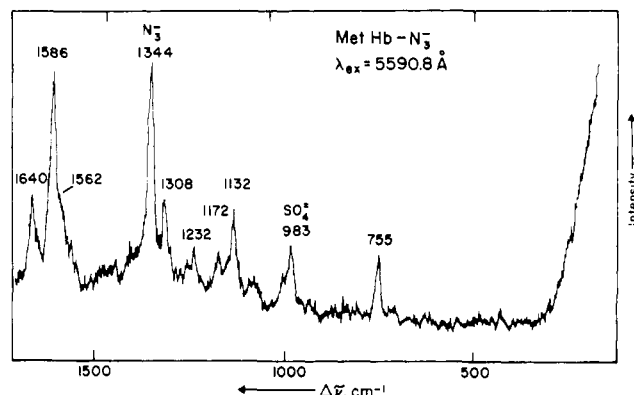
FIGURE 1: Resonance Raman spectrum of MetHb-N_3^- (0.76 mM heme) containing 0.25 M Na_2SO_4 as an internal standard: λ_{ex} 5590.8 Å; energy 10^{-3} J/pulse; pulse repetition rate 30 Hz; scan speed 23 Å/min; slit width 3 Å. Positions of Raman lines due to SO_4^{2-} and uncomplexed azide are shown in the figure.

FIGURE 2: Resonance Raman spectrum of MetHb-N_3^-: λ_{ex} 6383.2 Å; conditions as in Figure 1.

Experimental Procedure

Human hemoglobin A_0 was purified by the method of Williams and Tsay (1973). Methemoglobin was prepared by oxidation of hemoglobin with excess potassium ferricyanide, followed by extensive dialysis against 0.1 M Hepes,[1] containing 1 mM EDTA, pH 7.0. Azide and fluoride complexes were formed by the addition of buffered solutions of the sodium salts directly to the capillary tubes to be used for Raman excitation.

The absorption spectra of diluted samples were measured on a Cary 118 recording spectrophotometer to confirm complete ligation. In addition, absorption spectra were measured for some of the Raman illuminated samples to determine if sample degradation had occurred; these were measured with thin films of the material spread on a microscope slide and held in place by a cover slip. No degradation was detected.

The $^{18}OH^-$ and $^{16}OH^-$ derivatives of MetHb used for the Raman spectra in Figure 7 were prepared by freeze-drying a buffered solution of MetHb-H_2O at about -60 °C. The freeze-dried material was redissolved in $H_2^{16}O$ or $H_2^{18}O$ (95% enriched in ^{18}O, Bio-Rad Lab., Richmond, Calif.). Each of the resulting solutions was slowly titrated to the alkaline pH value at 0 °C with small aliquots of a 30% solution of $Na^{16}OH$ in $H_2^{16}O$ with rapid stirring. Heme concentrations for the resonance Raman measurements were typically ca. 0.6 mM. Spectra were measured with and without 0.2 M Na_2SO_4 as an internal standard. No changes were observed in the Raman spectra on the addition of Na_2SO_4. The SO_4^{2-} line at 983 cm^{-1} is labeled in the spectra.

The Raman spectra were measured on an instrument constructed in the Chemistry Department of the University of California, Berkeley. The laser used to excite the Raman spectra is a CMX-4 xenon flashlamp-pumped dye laser (Chromatix Corp., Mountain View, Calif). The laser produces 1 μs pulses at a repetition rate between 5 and 30 Hz. The output of the laser was focused onto 1.5-mm i.d. melting-point capillaries which contained 10–50-μL volumes of the samples. The scattered light was collected by an ellipsoidal mirror and imaged into a Spex 1400 monochromator equipped with an EMI 9558QB photomultiplier. A polarization scrambler was placed
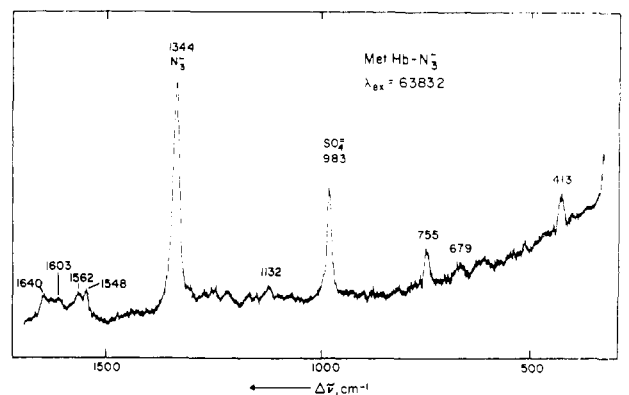
in front of the entrance slit of the monochromator to avoid intensity artifacts resulting from the polarization bias of the monochromator gratings. A quartz Wollaston prism was used to analyze the polarization of the scattered light.

The output of the Raman scattered light was detected and normalized to the intensity of the incident laser light by a dual-channel boxcar integrator. Prior to the sample, part of the incident laser beam was split off and was monitored by a second photomultiplier. The output of each electrometer was integrated with a 2-s time constant, and the integrated output of the sample photomultiplier was divided by that of the reference photomultiplier. Further details of the spectrometer are given elsewhere (Asher, 1976).

The spectrometer was calibrated with an Eppley Laboratories' standard incandescent intensity lamp. The excitation profiles were calculated from peak-height measurements of the Raman spectra and then normalized to the spectrometer efficiency curve and to the internal standard SO_4^{2-} line at 983 cm^{-1}. The Raman spectra themselves have not been normalized to the spectrometer efficiency profile, however. The excitation profiles have not been corrected for self-absorption; calculations similar to those of Strekas et al. (1974) indicate that the self-absorption phenomenon shifts the observed excitation profile maxima by at most a few nanometers. The major effect of the self-absorption correction is on the excitation profiles of the 1550- and 1610-cm^{-1} peaks of MetHb-F^-. The amplitude of the short-wavelength side of each of these excitation profiles is decreased, making the peaks somewhat more symmetric.

Results and Discussion

Methemoglobin Azide. Figures 1 and 2 show the resonance Raman spectra of the azide complex of MetHb excited at 5590.8 and 6383.2 Å, respectively. As the absorption spectrum of MetHb-N_3^- in Figure 3 shows, excitation at 5590.8 Å occurs between two absorption bands which have been assigned to the α and β bands of metalloporphyrins (Smith and Williams, 1970). Excitation at 6383.2 Å occurs within a weak absorption band at ca. 6400 Å, which has been assigned to a charge-transfer transition (Smith and Williams, 1970; Eaton and Hochstrasser, 1968).

The Raman spectrum shown in Figure 1 is similar to previously reported spectra of the azide complex of MetHb excited at 5682 Å between the α and β bands (Strekas and Spiro, 1972) and at 4416 in the Soret band (Yamamoto et al., 1973), and to the azide complex of metmyoglobin, excited at 4880 Å between the β and Soret band (Kitagawa et al., 1976). The dominant features in the resonance Raman spectrum excited at 5590.8 Å between the α and β bands (Figure 1) appear at

energies greater than 600 cm^{-1}. The bands occurring at 1640, 1586, 1308, 1132, and 755 cm^{-1} are due to porphyrin macrocyclic vibrational modes (Spiro, 1975). A comparison between the Raman spectrum of an aqueous solution of NaN$_3$ and a solution of MetHb-N$_3^-$ with lower concentrations of N$_3^-$ (not shown) indicates that the feature at 1344 cm^{-1} in Figures 1 and 2 is due to uncomplexed azide because the 1344-cm^{-1} peak does not appear at lower concentrations of NaN$_3^-$ (0.04 M), while the spectrum of the MetHb solution is still characteristic of the MetHb-N$_3^-$ complex.

The resonance Raman spectrum of MetHb-N$_3^-$ excited at 6383.2 Å in the charge-transfer band (Figure 2) shows a new Raman peak appearing at 413 cm^{-1}. A depolarization ratio measurement of the 413-cm^{-1} peak indicates that the peak is polarized, as is expected for an axial ligand stretch (Asher and Sauer, 1976), while an examination of the excitation profiles in Figure 3 shows that the 413-cm^{-1} peak is in resonance with only the charge-transfer band at 6400 Å. All of the other Raman peaks which appear upon 6383.2-Å excitation are more intense upon excitation at shorter wavelength and appear to be in resonance with the α and β bands.

The 413-cm^{-1} peak enhanced by excitation in the charge transfer band of MetHb-N$_3^-$ is close in energy to a vibration at 421 cm^{-1} observed in the IR spectrum of iron(III) octaethylporphine azide, which was assigned to the Fe-N$_3^-$ stretching vibration (Ogoshi et al., 1973). The unique enhancement of the 413-cm^{-1} peak by the charge-transfer band of MetHb-N$_3^-$, the close correspondence between the energies of the 413-cm^{-1} peak and the Fe-N$_3^-$ stretch observed in iron(III) octaethylporphine azide, the fact that the peak is polarized, and the selective appearance of the 413-cm^{-1} peak in the resonance Raman spectrum of MetHb-N$_3^-$ in contrast to its absence in MetHb-OH$^-$ and MetHb-F$^-$ (vide infra) all suggest that the 413-cm^{-1} peak results from a vibration of the azide nitrogen against the heme iron.

In contrast to the Raman spectra obtained from MnETP excited in the charge-transfer band (Asher and Sauer, 1976; Shelnutt et al., 1976; Gaughan et al., 1975), the porphyrin macrocyclic vibrational modes do not show an excitation profile maximum within the charge-transfer band of MetHb-N$_3^-$. In MetHb-N$_3^-$ the peaks resulting from porphyrin macrocyclic modes appear to derive their intensity from the α and β bands. Even the pyrrole–nitrogen–iron vibrations are not visibly enhanced, suggesting that the electronic transition which is responsible for the absorption band at 6400 Å in MetHb-N$_3^-$ is different from the electronic transition which is responsible for the charge-transfer band in MnETP. This conclusion is supported by polarization studies of the absorption spectra of single crystals of MetHb-N$_3^-$ (Kabat, 1967) and MetMb-N$_3^-$ (Eaton and Hochstrasser, 1968), which indicate that a z-polarized electronic transition is responsible for the absorption band at 6400 Å, and the MCD spectrum of MetMb-N$_3^-$ which shows a negative extremum due to the 6400-Å absorption band (Vickery et al., 1976). In contrast, the MCD spectrum of the charge-transfer band of MnETP exhibits a Faraday A term, indicating a degenerate, x, y polarized electronic transition (Boucher, 1972; Asher, 1976). Eaton and Hochstrasser assigned the ~6400-Å absorption band of MetMb-N$_3^-$ to either a porphyrin a$_{2u}(\pi) \rightarrow$ d$_{z^2}$ or an azide $(\pi) \rightarrow$ iron (d) charge-transfer band. It is less likely that the band results from a d $\rightarrow$ d transition, because its molar absorptivity (ϵ ~10^3) is an order of magnitude higher than the molar absorptivity expected for d $\rightarrow$ d transitions (Smith and Williams, 1970).

The Raman data do not distinguish the a$_{2u}(\pi) \rightarrow$ d$_{z^2}$ from the azide $(\pi) \rightarrow$ iron (d) charge-transfer transition. In-plane

porphyrin and pyrrole–nitrogen–Fe^{3+} vibrations may not couple well to a z-polarized electronic transition. However, enhancement of out-of-plane vibrations involving the Fe^{3+}–pyrrole linkages would be expected. Unfortunately, the magnitude of this enhancement is difficult to predict. No new features which might be due to out-of-plane vibrations of the heme are observed in the Raman spectrum of MetHb-N$_3^-$. The vibration of the Fe-N$_3^-$ linkage is expected to be the major vibration enhanced in an a$_{1u}$ or a$_{2u} \rightarrow$ d$_{z^2}$ transition, since the azide is bound by the d$_{z^2}$ orbital of the iron (Kitagawa et al., 1976).

A charge-transfer transition from the azide π orbitals to the d orbitals of the iron would be expected to enhance the Raman peak due to the vibration of the Fe-N$_3^-$ linkage. However, internal vibrations of the N$_3^-$ might also be enhanced, since the enhancement of an asymmetric azide stretch has been observed in the resonance Raman spectra of azidomethemerythrin (Dunn et al., 1975), and an analogous enhancement of pyridine vibrations occurs upon excitation in a Fe$^{2+} \rightarrow$ pyridine
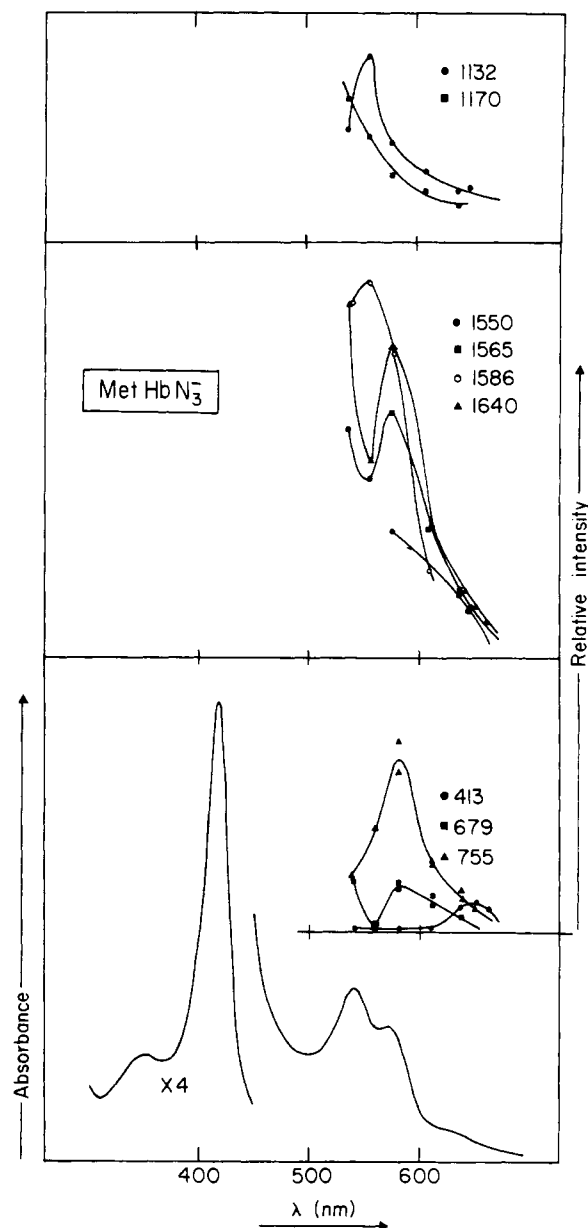

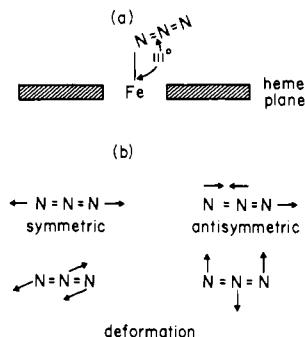FIGURE 3: Absorption spectrum and excitation profiles of MetHb-N$_3^-$. The intensities of the Raman lines were corrected for the spectrometer response and then normalized against the SO$_4^{2-}$ line at 983 cm^{-1}.

FIGURE 4: (a) Geometry of the heme-azide complex of MetHb-N$_3^-$ (Stryer et al., 1964). (b) Vibrational modes of N$_3^-$.
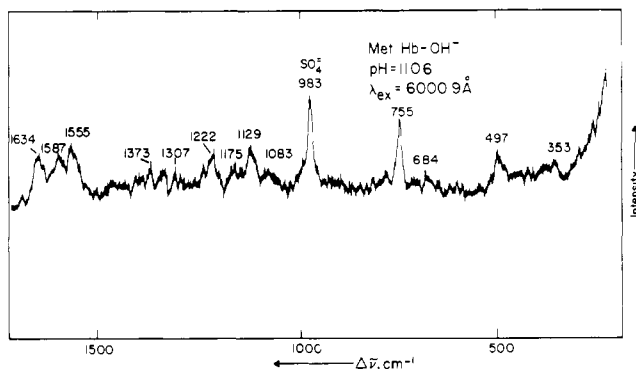

FIGURE 5: Resonance Raman spectrum of MetHb-OH$^-$ (0.76 mM heme), pH 11.06. Contains 0.2 M Na$_2$SO$_4$ as an internal standard: λ_{ex} 6000.9 Å; energy 4 × 10^{-3} J/pulse; pulse repetition rate 30 Hz; scan speed 23 Å/min; slit width 2.5 Å.

FIGURE 6: Absorption spectra and excitation profiles of methemoglobin-OH$^-$. Conditions were the same as in Figure 3.

charge-transfer band at 4765 Å in pyridine complexes of iron(II) mesoporphyrin IX (Spiro and Burke, 1976). Raman spectra of MetHb-N$_3^-$ with lower concentrations of N$_3^-$ (0.04 M) show spectra similar to Figure 2. The only difference is the disappearance of the 1344-cm^{-1} peak, due to free N$_3^-$. There is no obvious enhancement of internal azide vibrations upon excitation in the charge-transfer band at 6400 Å.

The lack of enhancement of internal azide vibrations may be due to the geometry of the heme-azide complex. It is known from x-ray crystallographic studies of MetMb-N$_3^-$ that azide binds at an 111° angle to the normal of the heme plane (Stryer et al., 1964), Figure 4. Both the symmetric and antisymmetric vibrations of the N$_3^-$ are at 111° to the z-polarized electronic transition. Thus, these azide vibrations may not couple well with the z-polarized charge-transfer transition. It is difficult to predict the enhancement expected for the doubly degenerate azide-deformation mode. Another possibility is that the charge-transfer transition occurs from a nonbonding orbital of the azide to a d$_{z^2}$ orbital of the iron. This transition, which is z polarized, would enhance the vibration between the iron and azide nitrogen, but neither the pyrrole-nitrogen-iron nor internal azide vibrations would be appreciably enhanced.

Methemoglobin Hydroxide. Figure 5 shows the resonance Raman spectrum of MetHb-OH$^-$ at pH 11.06 excited at 6000.9 Å. Dominant features in the spectrum occur at 497, 755, 1555, 1587, and 1634 cm^{-1}. A number of differences appear between the resonance Raman spectrum of MetHb-OH$^-$ and MetHb-N$_3^-$. The 413-cm^{-1} peak seen for the azide complex is absent in the hydroxide form but a new peak is found at 497 cm^{-1}. An examination of the absorption spectrum and the excitation profiles presented in Figure 6 indicates that the 497-cm^{-1} peak is in resonance with the shoulder near 6000 Å in the absorption spectrum. The other Raman bands increase
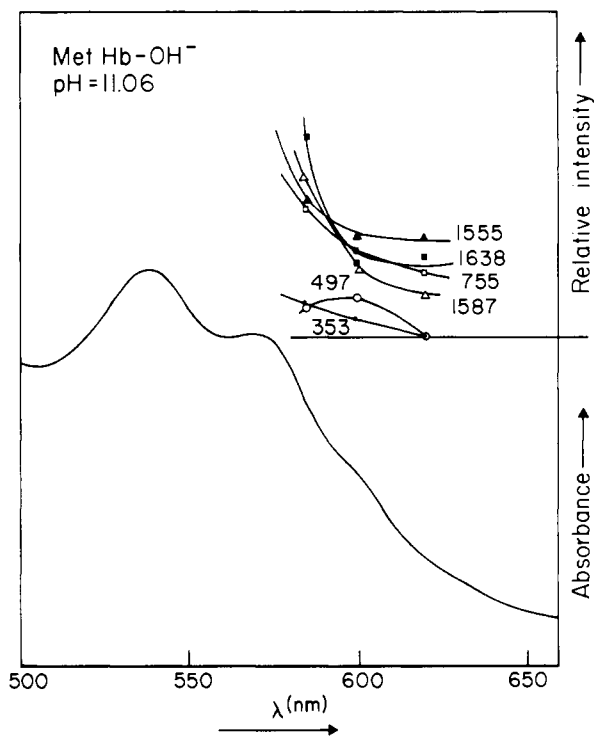
dramatically in intensity as the excitation wavelength decreases, indicating that they are resonance enhanced by the 5700- and/or 5400-Å π to π^* absorption bands.

The unique enhancement of the 497-cm^{-1} band by the shoulder at 6000 Å is similar to the selective enhancement shown by the 413-cm^{-1} Raman peak in the charge-transfer band of MetHb-N$_3^-$ at 6400 Å. To determine whether the 497-cm^{-1} peak results from the Fe-O stretch, Raman spectra were obtained for MetHb-^{18}OH. Figure 7 shows the resonance Raman spectra of MetHb-^{18}OH and MetHb-^{16}OH excited at 6004.7 Å. The 497-cm^{-1} peak which appears in the spectrum of MetHb-^{16}OH$^-$ shifts 20 cm^{-1} to lower energy following substitution by ^{18}OH$^-$. The increased background in both of the spectra in Figure 7 over that in Figure 5 presumably is due to some denaturation of the MetHb during the freeze-drying process.

Using a harmonic oscillator model, the energy shift for the 497-cm^{-1} peak is only 2 cm^{-1} less than the 22-cm^{-1} shift predicted if this vibration were due to a pure Fe-O stretch, indicating little mixing with other vibrational modes. The enhancement of the Fe-O stretch at 6000 Å and the lack of observable excitation profile maxima for the other porphyrin macrocyclic modes in this region suggest that the shoulder of the absorption band at ca. 6000 Å is a pure charge-transfer transition which is not mixed with the π to π^* transitions of the porphyrin macrocycle. The similarity between the excitation profiles in MetHb-N$_3^-$ and MetHb-OH$^-$ suggests that the electronic transitions are similar charge-transfer transitions, and that the shoulder at ca. 6000 Å in MetHb-OH$^-$ is z polarized.

Other differences between the resonance Raman spectrum of MetHb-OH$^-$ and MetMb-N$_3^-$ occur in the spin-state-sensitive regions of the Raman spectrum between 1550 and 1640 cm^{-1} (Spiro, 1975; Spiro and Burke, 1976). In contrast to MetHb-N$_3^-$ which is predominantly low spin, MetHb-OH$^-$ exists in a spin-state equilibrium with comparable concentrations of the high- and low-spin forms (George et al., 1961).
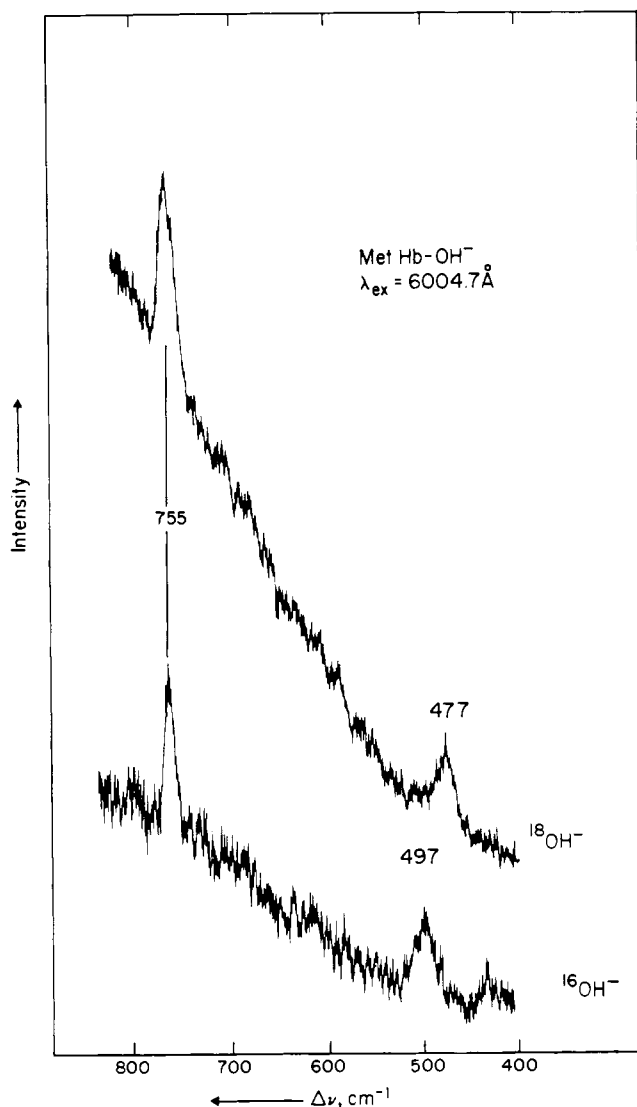
FIGURE 7: Resonance Raman spectra of MetHb-^{16}OH$^-$ and MetHb-^{18}OH$^-$ (ca. 0.9 mM heme): pH 10.2; λ_{ex} 6004.7 Å; energy 2 × 10^{-3} J/pulse; pulse repetition rate 30 Hz; scan speed 16 Å/min; slit width 4.5 Å.

FIGURE 8: Resonance Raman spectrum of MetHb-F$^-$ (0.76 mM heme) containing 0.25 M Na$_2$SO$_4$ as an internal standard: λ_{ex} 6175.1 Å; energy 2 × 10^{-3} J/pulse; pulse repetition rate 30 Hz; scan speed 23 Å/min; slit width 3.7 Å; p, polarized; dp, depolarized; ip, inversely polarized.

Thus, instead of the low-spin peaks at ca. 1640 and 1586 cm^{-1} in the Raman spectrum of MetHb-N$_3^-$ (Figure 1), a complex spectrum of overlapping peaks occurs between 1550 and 1640 cm^{-1} for MetHb-OH$^-$.

In contrast to MetHb-N$_3^-$ and MetMb-N$_3^-$ which show almost identical Raman spectra when excited in the same spectral region (Strekas and Spiro, 1973) and MetHb-F$^-$ and MetMb-F$^-$ (vide infra) which also show almost identical spectra, Raman spectra of the hydroxide complex of MetHb differ from those of MetMb in the 1550–1640-cm^{-1} region when excited at 4416 Å (Yamamoto et al., 1973), and when excited between 5800 and 6100 Å (S. Asher, L. Vickery, T. Schuster, and K. Sauer, unpublished observations). The differences in the Raman spectra presumably reflect a change in the spin-state equilibrium of MetHb-OH$^-$ from that of MetMb-OH$^-$ (Yamamoto et al., 1973; Ozaki et al., 1976). Differences between the heme electronic structure of MetHb-OH$^-$ and MetMb-OH$^-$ are also observed by magnetic-susceptibility measurements (George et al., 1961). The concentration of the high-spin form appears to be higher in MetMb-OH$^-$ than in MetHb-OH$^-$. In addition, absorption measurements show a decrease in absorption for MetMb-OH$^-$ between 5000 and 5800 Å, and an increase in absorption above
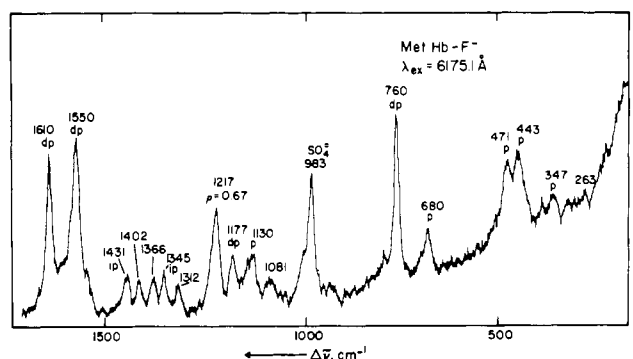
5800 Å with a peak appearing at ca. 6000 Å (George et al., 1961). Raman spectra of MetMb-OH$^-$, excited in the 6000-Å absorption band, show a dramatic enhancement of the peak corresponding to the Fe–O stretch, which becomes the dominant feature in the Raman spectrum (S. Asher, L. Vickery, T. Schuster, and K. Sauer, unpublished observations), suggesting an increased charge-transfer contribution to the absorption spectrum at ca. 6000 Å in MetMb-OH$^-$ over that of MetHb-OH$^-$. Additionally, the Fe–O stretch appears to be shifted to slightly lower frequency, indicating a weaker Fe–O bond in MetMb-OH$^-$ than in MetHb-OH$^-$.

Methemoglobin Fluoride. In contrast to MetHb-N$_3^-$ which is predominantly low spin and MetHb-OH$^-$ which is in a thermal spin-state equilibrium, MetHb-F$^-$ is almost purely high spin (Beetlestone and George, 1964). Figure 8 shows the resonance Raman spectrum of MetHb-F$^-$ excited at 6175.1 Å. The dominant features in the Raman spectrum occur at 1610, 1550, 1217, 760, 471, and 443 cm^{-1}. The higher frequency region of the Raman spectrum shown in Figure 8 (>700 cm^{-1}) is qualitatively similar to previously reported spectra of MetHb-F$^-$ excited at 6328 Å and between 4579 and 5145 Å (Strekas et al., 1973). In contrast to the Raman spectra of MetHb-N$_3^-$ and MetHb-OH$^-$, which show peaks at 413 and 497 cm^{-1}, respectively, intense low-frequency peaks in MetHb-F$^-$ appear at 443 and 471 cm^{-1}. An examination of the excitation profiles and the absorption spectrum of MetHb-F$^-$ shown in Figure 9 reveals a number of excitation profile maxima. The two peaks at 443 and 471 cm^{-1}, which are polarized, appear to be in resonance with the absorption peak at ~6000 Å. However, it should be noted that the 443-cm^{-1} peak shows an intensity maximum at a somewhat higher wavelength than does the 471-cm^{-1} peak, and at excitation wavelengths lower than 6080 Å the 471-cm^{-1} peak is more intense than the 443-cm^{-1} peak. The appearance of the 443- and 471-cm^{-1} doublet does not arise from subunit differences in MetHb-F$^-$ because a very similar Raman spectrum is observed when MetMb-F$^-$ is excited in this spectral region (S. Asher, L. Vickery, T. Schuster, and K. Sauer, unpublished observations).

In view of the enhancement of Fe–axial ligand vibrations by the charge-transfer bands of MetHb-N$_3^-$ and MetHb-OH$^-$, the fact that the peaks are polarized, as is expected for axial ligand vibrations (Asher and Sauer, 1976), and the fact that intense vibrations between 400 and 500 cm^{-1} have not been observed for any metalloporphyrins other than MnETP-F$^-$ (495 cm^{-1}, Asher and Sauer, 1976), MetHb-N$_3^-$, MetMb-OH$^-$, and MetHb-OH$^-$, it is tempting to assign the 443- and/or 471-cm^{-1} peaks to the Fe–F stretch. However,
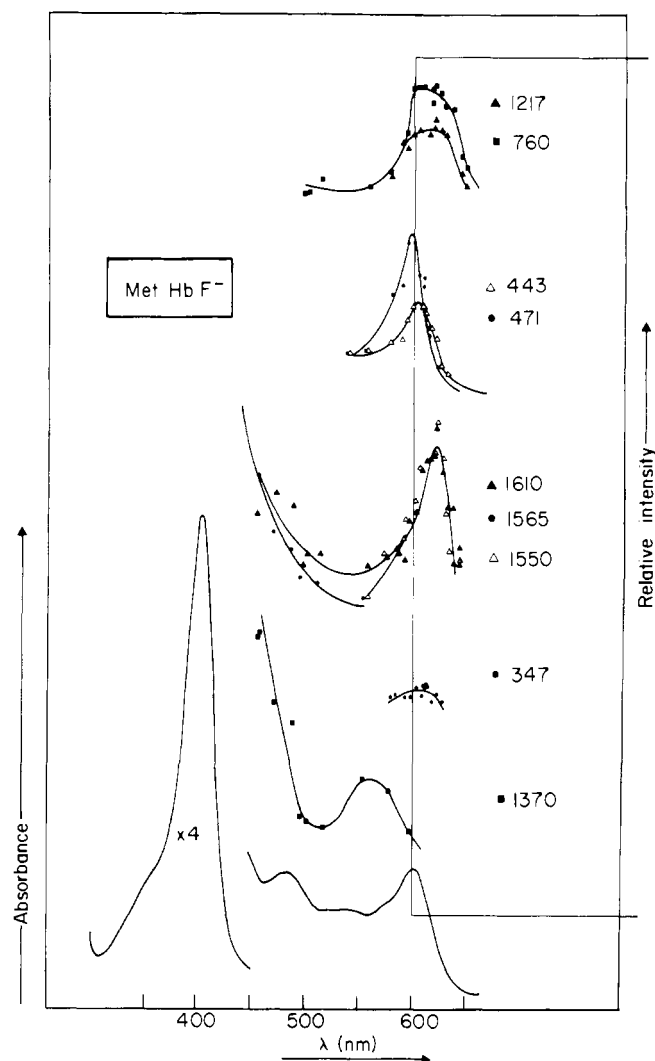
FIGURE 9: Absorption spectrum and excitation profiles of MetHb-F^-. Conditions were as in Figure 3. The points at wavelengths below 5200 Å were obtained from previously reported spectra (Strekas et al., 1973).

Ogoshi et al. (1973), in their IR studies of metalloporphyrins, assigned an Fe-F^- stretch in ferric octaethylporphine fluoride to a band at 602 cm^{-1}, while Kincaid and Nakamoto (1976) assigned the Fe-F stretch to a 600-cm^{-1} peak in the resonance Raman spectrum of the F^- complex of ferric octaethylporphine, and Spiro and Burke (1976) observed the selective appearance of a 580-cm^{-1} peak in the resonance Raman spectrum of the fluoride complex of ferric mesoporphyrin IX dimethyl ester.

The environment of the iron in MetHb-F^-, however, is much different from that in fluoride complexes of non-protein-bound metalloporphyrins. In 5-coordinate, high-spin complexes of ferric porphyrins, the iron lies ~0.45 Å out of the plane toward the 5th axial ligand (Hoard, 1975). However, in MetHb-F^- the iron lies 0.3 Å out of the plane displaced toward the proximal histidine on the opposite side from which the F^- must bind (Deatherage et al., 1976b; Perutz et al., 1974a). Thus, the iron is displaced about 0.75 Å in MetHb-F^- compared to ferric porphyrin fluoride. The ~120-cm^{-1} decrease in the frequency of the Fe-F^- stretch in MetHb-F^- from that in non-protein-bound F^- complexes of ferric metalloporphyrins may thus result from nonbonding interactions between the charge cloud of the F^- and the orbitals of the pyrrole nitrogens. This is represented diagrammatically in Figure 10. Assuming an equilibrium bond length of 1.97 Å for Fe-F, i.e., the sum of
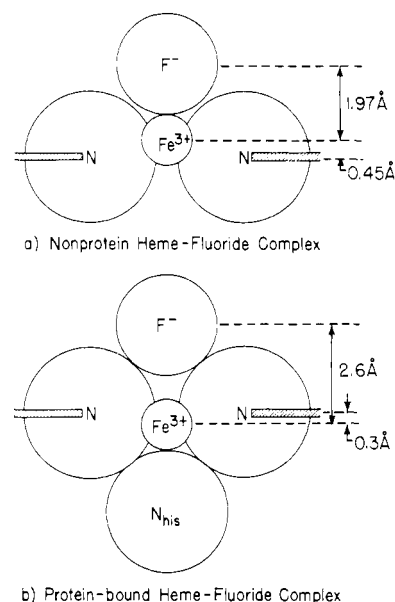
their ionic radii, little steric interaction would be expected to occur between the van der Waals radii of the pyrrole nitrogens (1.70 Å, Hoard, 1975) and the ionic radius of the F^- in iron-(III) porphyrins in which the Fe is out-of-plane toward the F^- atom. In MetHb-F^- and MetMb-F^-, however, the steric constraints imposed by the nitrogen π orbitals would be expected to cause an elongation of the bond and a decrease in the frequency of the vibration. If the orbitals were hard spheres the bond would elongate by 30% to ~2.6 Å. The Fe-F^- bond appears to be like a stretched spring. The ~120 cm^{-1} decrease for the 0.75 Å movement of the Fe atom through the heme plane suggests that this vibration should provide a sensitive measure of any movement of the iron with respect to the porphyrin plane. Assuming a linear decrease in the frequency of the Fe-F vibration with increasing bond length, a 1.7-cm^{-1} shift is expected for a change of 0.01 Å in the out-of-plane distance of the iron atom.

The existence of the two polarized, adjacent peaks at 471 and 443 cm^{-1}, which have similar excitation profiles, suggests a correlation between them. The observation by Deatherage et al. (1976b), using x-ray difference Fourier diffraction, that the environment of the F^- ion in MetHb-F^- is heterogeneous may account for the presence of two peaks assignable to the Fe-F^- stretch. Deatherage et al. (1976b) observed the presence of a previously unnoticed feature within the heme cavity which was proposed to be a water molecule stabilized by hydrogen bonding to the fluoride ion in the heme cavities of both the α and β subunits. The magnitude of the feature suggested that the water molecule is stabilized in the heme pocket only part of the time, leading to a heterogeneity in the fluoride environment. Hydrogen bonding of water with the fluoride ion should decrease the frequency of the Fe-F^- vibration. Thus, the 471-cm^{-1} peak may correspond to an unperturbed Fe-F^- stretch, while the 443-cm^{-1} peak may correspond to the Fe-F^- stretch shifted to lower energy due to hydrogen bonding of water to fluoride.

The excitation profiles of the resonance Raman peaks of MetHb-F^- (Figure 9) show a more complicated pattern than

those of MetHb-OH$^-$ and MetHb-N$_3^-$, presumably because of the complexity of the visible absorption spectrum, which consists of at least four overlapping bands (Eaton and Hochstrasser, 1968). It has been proposed that the complexity of the visible absorption spectrum of MetHb-F$^-$ results from the mixing of charge-transfer bands with porphyrin $\pi \rightarrow \pi^*$ transitions to the extent that none of the absorption bands in the visible region can be considered as pure transitions (Zerner et al., 1966; Eaton and Hochstrasser, 1968; Smith and Williams, 1970).

In addition to the 443- and 471-cm^{-1} peaks, a weak, polarized Raman peak at 347 cm^{-1} appears to be in resonance with the 6000-Å absorption band maximum. The vibrations which show their maximum intensity at 6250 Å occur at 1610 (dp) and 1550 (dp) cm^{-1} (Figure 9). Since these peaks are depolarized, they are either of B$_{1g}$ or B$_{2g}$ symmetry in the D_{4h} point group. However, both the 760- (dp) and the 1217-cm^{-1} peaks show broad excitation profiles, suggesting that the excitation profiles (Figure 9) may result from the overlap of two maxima at 6000 and 6250 Å. Weaker, anomalously polarized peaks at 1345 and 1431 cm^{-1} appear upon excitation at ca. 6000 and 6250 Å. However, these peaks are not observed with excitation between 6080.8 and 6150.0 Å. The 1217-cm^{-1} peak shows a depolarization ratio, ρ, of 0.62 when excited at 6147.1 Å. However, with excitation at 6328 Å the 1217-cm^{-1} peak is found to be depolarized (Strekas et al., 1973). For in-plane electronic transitions in the D_{4h} point group, theory predicts that vibrations of A$_{1g}$ symmetry have $\rho = 0.125$, those of A$_{2g}$ symmetry have $\rho = \infty$, and those of B$_{1g}$ or B$_{2g}$ have $\rho = 0.75$ (Pezolet et al., 1973). A depolarization ratio intermediate between 0.125 and 0.75 suggests an overlap of an A$_{1g}$ vibration with a vibration of B$_{1g}$, B$_{2g}$, or A$_{2g}$ symmetry. Thus, it appears that only depolarized or inversely polarized peaks show an intensity maximum at 6250 Å.

In their measurements of the single crystal polarized absorption spectrum of MetMb-F$^-$, Eaton and Hochstrasser observed that in contrast to MetMb-N$_3^-$, which shows a z-polarized transition at ca. 6400 Å, the entire visible absorption spectrum of MetMb-F$^-$ was x, y polarized. However, they noted that an inequivalency of x- and y-polarized electronic transitions occurred at ca. 6250 Å, indicating a splitting of the degeneracy of the x and y directions. Since an inequivalency of the x and y directions occurs at 6250 Å, a description of the electronic transitions in the D_{2h} point group is appropriate. In the D_{2h} point group the symmetry of the vibrations which would mix x and y electronic transitions is:

$$\Gamma_x \times \Gamma_y = \Gamma_{B_{2u}} \times \Gamma_{B_{3u}} = \Gamma_{B_{1g}}$$

Thus, vibrations of B$_{1g}$ symmetry are expected to be enhanced at 6250 Å. However, B$_{1g}$ vibrations in the D_{2h} point group correlate to B$_{2g}$ and A$_{2g}$ vibrations in the D_{4h} point group (Kitagawa et al., 1975), and depolarized and inversely polarized vibrations are expected to be enhanced at 6250 Å, in agreement with the excitation profile data.

Eaton and Hochstrasser suggested that the inequivalency of the x and y directions results from the splitting of the d$_{xz}$, d$_{yz}$ orbitals of the iron. However, the exclusive enhancement at 6250 Å of porphyrin macrocyclic modes suggests that the origin of the degeneracy splitting lies mainly in the porphyrin macrocycle and is not a result of an axial ligand-induced inequivalency of the d$_{xz}$ or d$_{yz}$ orbitals. If the inequivalency resulted from the metal orbitals, vibrations of A$_{2g}$ and B$_{2g}$ symmetry about the metal would be enhanced by terms such as $\langle d_{xz} \| \partial H/\partial Q_a \| d_{yz} \rangle$ in the polarizability expression (Albrecht, 1961; Asher and Sauer, 1976). Thus, the inequivalency

TABLE I: Raman Vibrations Assigned to Fe-X$^-$ Stretch in MetHb (X = N$_3^-$, OH$^-$, F$^-$).

Derivative	$\Delta\nu$ (cm^{-1})	Remarks
MetHb-N$_3^-$	413	
MetHb-^{16}OH	497	
MetHb-^{18}OH	477	
MetHb-F$^-$	471	Fe-F
	443	Fe-F[a]

[a] Fluoride anion hydrogen bonded to water.

in the x and y directions may result from an interaction not through the iron but directly on the porphyrin plane by the heme environment. This could result from a steric influence of the proximal histidine, which might bind to the iron in one particular orientation with respect to the x and y directions of the porphyrin macrocycle. Alternatively, the splitting might result from the interaction of the heme with another species in the heme cavity.

Table I summarizes the Fe-ligand peaks assigned in this report.

Effect of Inositol Hexaphosphate. Inositol hexaphosphate (IHP), which shifts the spin-state equilibrium of MetHb-OH$^-$ and MetHb-N$_3^-$ to favor the high-spin form, appears to convert MetHb-F$^-$ from the R to the T form (Perutz et al., 1974a,b). For the hydroxide and azide derivatives, this spin change should be reflected in an increase in the time-averaged distance of the iron from the heme plane; a similar relative movement of the iron is expected in the fluoride derivative, since Perutz et al. (1974b) propose that in the T form the iron lies further out of the porphyrin plane than in the R form. The steric interactions of the sixth ligand with the heme plane suggest that the frequency of the vibration of the sixth ligand to the iron should be a sensitive function of the out-of-plane distance of the metal.

IHP was added to solutions of MetHb-X (X = F$^-$, OH$^-$, N$_3^-$), and resonance Raman spectra were excited at wavelengths which maximally enhanced the Fe-X vibrations. The addition of IHP to solutions of MetHb-X$^-$ had no effect on the entire resonance Raman spectra within the signal-to-noise ratio of the spectra. Frequency shifts of 3 cm^{-1} or changes in peak intensity of 10% should have been readily detected. This lack of an effect of IHP on the porphyrin macrocyclic modes of MetHb-F$^-$ was previously noted by Szabo and Barron (1975).

The fact that IHP has no effect on the energy of the Fe-O vibration in MetHb-OH$^-$ may simply reflect the fact that IHP does not bind well to MetHb at pH greater than 7, and the lack of a detectable effect on MetHb-N$_3^-$ may be due to the fact that the IHP-induced spin-state changes in MetHb-N$_3^-$ are quite small (Perutz et al., 1974b). However, the lack of a shift in the energy of the vibrations which are assigned to Fe-F$^-$ stretching suggests that little, if any, movement of the iron occurs on the addition of IHP. Based on the discussion given in the preceding section suggesting a 1-2 cm^{-1} shift for a change of 0.01 Å in the iron-ligand bond distance in MetHb-F$^-$, we can conclude that there is little or no movement of the iron atom with respect to the heme plane. This analysis, of course, assumes the correctness of our assignment of either the 471- or 443-cm^{-1} peak to an Fe-F$^-$ stretch. A comparison of the extended x-ray absorption fine-structure spectrum (EXAFS) of deoxyHb A with that of deoxyHb Kempsey also led Eisenberger et al. to conclude that there was no substantial movement (<0.02 Å) of the iron between the high- (R) and

the low-affinity (T) quaternary forms (Eisenberger et al., 1976).

It has been suggested that a steric effect of the protein on the heme decreases the accessibility of the heme-binding site in the T form (Perutz et al., 1976; Deatherage et al., 1976a). It has been proposed that a change occurs in the π interactions between the porphyrin macrocycle and the surrounding protein matrix (Maxwell and Caughey, 1976), upon the R to T transition, leading to changes in the electronic structure of the porphyrin, concomitant changes in the iron d orbitals and changes in the ligand affinities of the heme. The energy of the Fe–axial ligand vibrations reported here may not be a good monitor of these effects. However, it is possible that the excitation profiles may contain some information on steric and π nonbonding interactions between the protein and the heme, or both. Polarized single crystal absorption spectra and the excitation profile data both indicate an inequivalency of the x and y directions of MetHb-F$^-$ at 6250 Å. If the inequivalency of the x and y directions results from nonbonding interactions between the heme and the protein, changes in the excitation profiles upon the addition of IHP may monitor differences in the interactions between the R and T quaternary forms.

Note Added in Proof

A report on the x-ray difference map of an IHP complex of human MetHb-F$^-$ against horse deoxyHb has appeared since this paper was submitted (Fermi, G., and Perutz, M. F. (1977), *J. Mol. Biol. 114*, 421). It concluded that either the iron atoms of the α chains are up to 0.8 Å to the distal side of the heme plane in the IHP complex of MetHb-F$^-$ or the iron atoms are in or near the porphyrin plane and that "the relative positions of the Fe, N$_\epsilon$ and the porphyrin plane might be similar in the R and T state fluoromethemoglobins". Our results, which indicate that no movement of the iron occurs upon the addition of IHP to MetHb-F$^-$, are consistent with their second interpretation: that the positions of the iron atoms in the R and T forms of MetHb-F$^-$ are almost identical and the iron is close to in-plane. Even if the iron atoms are in-plane, the iron–fluoride bond must stretch by 15% to avoid penetration of the ionic radius of the fluoride anion with the van der Waals radii of the pyrrole nitrogens. Thus, the vibrational frequency of the iron–fluoride bond would still be expected to be a sensitive monitor of iron movement.

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