Dependence of Axial Ligand Force Constants upon Globin Structure in Hemoglobin and Myoglobin

A Resonance Raman Study

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Cooperativity in hemoglobin ligand binding depends upon the ability of the hemoglobin tetramer to undergo a transition between different quarternary conformations (labeled R and T) that have different ligand affinities (for recent reviews see Perutz, 1979; Moffat et al., 1979). The relevant molecular question is how are the heme ligand affinities modulated by tertiary and quarternary protein structural changes. Possible mechanisms for decreasing ligand affinities include increases in the out-of-heme-plane iron distances (thereby preventing strong ligand binding) or a blocking of the sixth axial ligand site by residues on the distal side of the heme plane. In either case, very small structural alterations corresponding to shifts in atomic positions of a fraction of an angstrom are expected to be significant. Recently, we used resonance Raman spectroscopy to examine the protein structural dependence of iron-axial ligand (Fe—X) vibrational frequencies in the F^- and OH^- derivatives of methemoglobin (Hb^{III} and metmyoglobin (Mb^{III}), demonstrated the sensitivity of the Fe-ligand stretching frequencies to small changes in heme geometry, and proposed quantitative relations between the Fe—X stretching frequencies, molecular bond lengths, and iron-out-of-heme-plane distances (Asher and Schuster, 1979).

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In this report, we examine differences in heme geometry between the α and β subunits of $Hb^{III}F$. We also examine interactions between the F^- ligand and amino acid residues on the distal side of the heme plane. We observe directly for the first time protonation of the distal histidine and the resulting hydrogen bond formed by the protonated distal histidine with the F^- ligand.

Experiment

Sperm whale myoglobin was obtained in the lyophilized met form from Miles-Seravac (Batch 15). It was purified on a CM-50-Sephadex column resin according to the method of Hapner et al. (1968). The principal Mb component (fraction 4) was used for the present study and the protein was stored as the salt-free lyophilized protein. The major component of human hemoglobin, A_0, was purified as oxyhemoglobin by the method of Williams and Tsay (1973). Methemoglobin was prepared by oxidation of oxyhemoglobin with excess potassium ferricyanide followed by extensive dialysis against double-distilled water and stored as 3% solutions in liquid N_2. The α and β hemoglobin subunits were prepared in the oxygenated forms from human Hb A_0 by the method of Bucci and Fronticelli (1965) and the blocked-SH groups were regenerated by the method of Geraci et al. (1969). Methemoglobin and metmyoglobin fluoride samples were prepared by addition of the purified protein to buffer solutions followed by addition of the appropriate amount of KF. The α

and β subunit fluoride derivatives were prepared by oxidation of the oxy chains with $K_3Fe(CN)_6$ in the presence of buffer and KF.

The $Hb^{III}F$ and $Mb^{III}F$ solutions at different pH values were prepared by dissolving the appropriate amount of the protein in buffer solution close to the desired pH value and titrating with 0.1 N HCl or KOH with rapid stirring to the exact pH desired.

The Raman spectra were measured using a microcomputer interfaced Raman spectrometer described in detail elsewhere (Asher and Schuster, 1979), and using a rotating cell to avoid sample decomposition. The Raman difference spectra were obtained by direct numerical subtraction of the original data.

Results

Figure 1 shows the resonance Raman spectra of the isolated chains and their difference spectra. The high-frequency region of the Raman spectra contains porphyrin macrocyclic modes at 1,550 and 1,610 cm^{-1}, indicating a high-spin iron (Spiro, 1975; Spiro and Burke, 1976; Kitigawa et al., 1976). The 983-cm^{-1} peak is contributed by SO_4^{2-} which we added as an internal standard in the form of Na_2SO_4. The doublet at approximately 440 and 470 cm^{-1}, which we have previously assigned to Fe—F stretching vibrations (Asher and Schuster, 1979; Asher et al., 1977) are enhanced in these spectra because resonance excitation is within the 6000-Å charge transfer absorption bands of these derivatives (Asher and Sauer, 1976). The ~470-cm^{-1} component results from an unperturbed Fe—F stretch, whereas the 440-cm^{-1} component results from a heterogeneity within the heme cavities resulting from a partial occupancy of water in some of the crevices (Deatherage et al., 1976); the water molecule hydrogen-bonds to the F^- lowering its stretching frequency (Asher and Schuster, 1979; Asher et al., 1977).

The difference spectrum clearly indicates frequency shifts for many of the Raman peaks between the α and β chains. The α subunits ~760, ~1,550, and ~1,610 cm^{-1} porphyrin macrocyclic vibrational modes are higher in frequency than those of the β subunits. In contrast, the α subunit Fe—F doublet at 466 and 441 cm^{-1} occurs ~5 cm^{-1} lower in frequency than does the β subunit doublet. By directly shifting the individual spectra numerically, it appears that the 471- and 444-cm^{-1} β subunit doublet rigidly shifts by 5 cm^{-1} to lower frequency in the α subunit.

We examined changes in the heme conformation within the α and β subunits induced by their recombination into tetrameric $Hb^{III}F$. Figure 2 showing the resonance Raman spectra of $Hb^{III}F$ is identical to spectra obtained from recombined isolated α and β met fluoride subunits. The lower

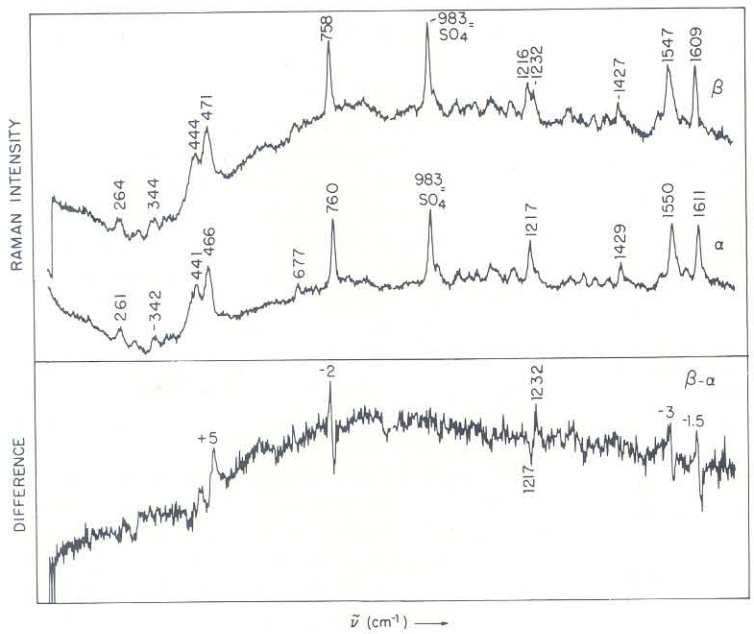


Figure 1. Resonance Raman spectra of isolated α and β chains and their difference spectrum. $\lambda_{ex} = 6{,}050.6$ Å. Average slitwidth = 4 cm^{-1}. Integration time = 1 sec. Power = 0.37 W. Heme concentration = 0.255 mM. 0.2 M Na_2S04, 0.2 M Hepes buffer, pH = 7.0, 0.1 M KF, 1.0 mM EDTA. The 983-cm^{-1} peak results from the internal standard SO_4^{2-} vibrational mode.

part of Figure 2 shows a difference spectrum between the tetramer and the sum of the spectra of the isolated chains. The difference spectrum was calculated by scaling the intensities of the spin-state sensitive Raman peaks in the spectral sum of the isolated chains such that they were identical to that in the tetramer. The scaling factor was within 10% of that necessary to account for differences in heme concentration and the number of spectral scans. The 983-cm^{-1} trough in the difference spectrum (Figure 2) results from an increased relative SO_4^{2-} concentration in the isolated chain samples compared to the tetramer sample.

The difference spectrum is essentially devoid of features except for the spectral region of the Fe—F stretching vibrations. A 23 ± 10% decrease in the Raman cross section of the Fe—F stretches in $Hb^{III}F$ compared to the isolated chains is necessary to account for the two small troughs in the 400–500-cm^{-1} difference spectra. The difference spectrum also indicates a small frequency shift for the average α and β Fe—F stretching vibrations from that of the tetramer. The observed Fe—F stretching frequency in $Hb^{III}F$ (which is the average of the Fe—F stretching frequencies of the α and β subunits) is decreased by ~ 1 ↑ cm^{-1} from the average value of the Fe—F stretching frequencies in the isolated α and β chains. Although the data indicate measurable differences in the Fe—F force constants between the isolated chains, the data cannot resolve whether this α and β chain difference is identically preserved in the tetramer. However, it is clear that the different intersubunit contacts existing in the tetramer compared to that in the isolated subunits results in only small changes in the average force constant of the Fe—F stretches in $Hb^{III}F$ from that occurring in the isolated chains.

We have examined the pH dependence of the absorption and resonance Raman spectra of $Mb^{III}F$ and $Hb^{III}F$. The absorption spectra of both of these proteins at high pH are indicative of formation of the hydroxide derivatives (not shown). Competitive OH^- binding at high pH in Mb^{III} solutions containing 0.1 M KF is clearly evident from the ~490-cm^{-1} shoulder on the high frequency of the 461-cm^{-1} Fe—F stretching peak (Figure 3); the Fe—O stretching vibration occurs at 490 cm^{-1} in $Mb^{III}OH$ (Asher and Schuster, 1979).

As the pH values of $Mb^{III}F$ and $Hb^{III}F$ solutions are decreased from neutrality the 6,000-Å charge transfer bands broaden somewhat and shift by ~5 nm to longer wavelengths. As shown in Figures 3 and 4, dramatic changes occur in the resonance Raman spectra of $Mb^{III}F$ and $Hb^{III}F$ at pH values below 6.0. At low pH peaks appear at 399 and 407 cm^{-1} in $Mb^{III}F$ and $Hb^{III}F$ simultaneously with a decrease in intensity for the 461- and 468-cm^{-1} $Mb^{III}F$ and $Hb^{III}F$ Fe—F stretching vibrations. The peaks above 600 cm^{-1} caused by in-plane porphyrin macrocyclic vibrational modes show little or no dependence upon sample pH; the selective Fe—F stretching frequency pH dependence is clearly evident from the $Mb^{III}F$ and $Hb^{III}F$ pH difference spectra shown in Figures 5 and 6.

The $Mb^{III}F$ difference spectrum shown in Figure 5 between pH 8.0 and 5.4 is devoid of

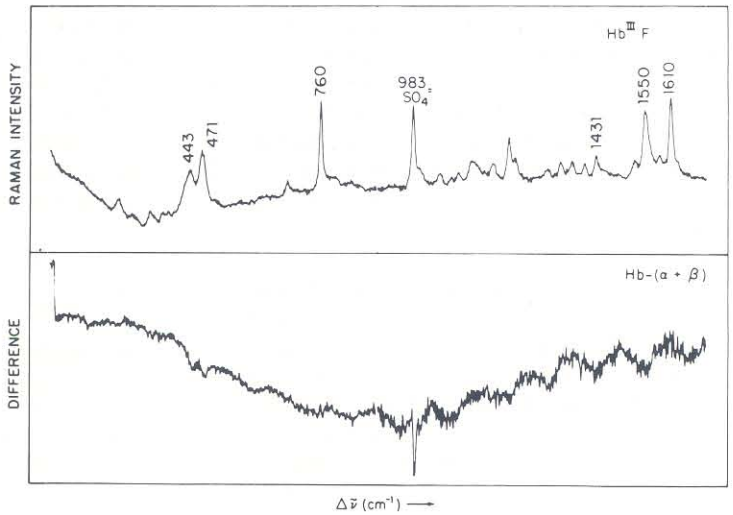


Figure 2. Resonance Raman spectrum of $Hb^{III}F$ and the difference spectrum between tetrameric $Hb^{III}F$ and the sum of the spectra of the isolated chains. $Hb^{III}F$ heme concentration = 0.261 mM. Conditions as in Figure 1. The 983-cm^{-1} trough in the difference spectrum results from an increased relative SO_4^{2-} concentration in the isolated subunits compared to the tetramer sample.

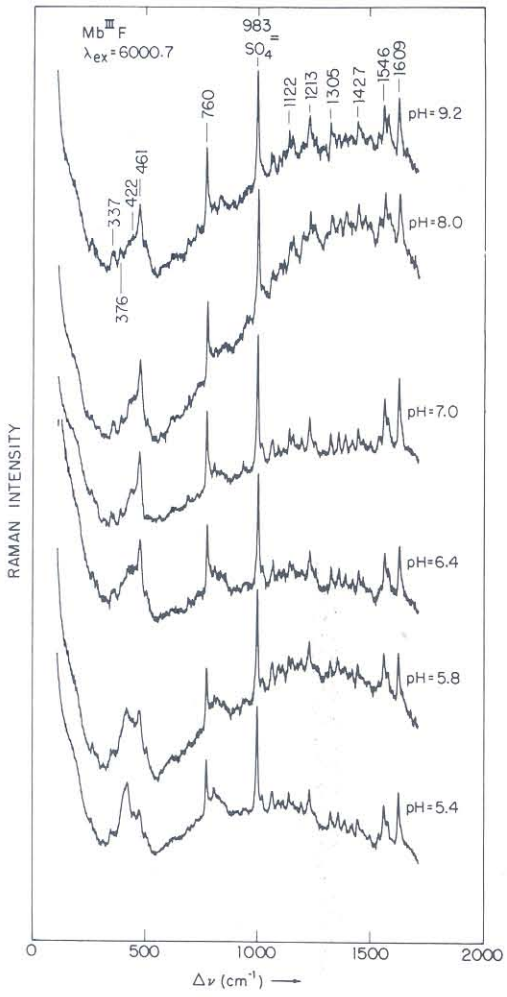


Figure 3. pH dependence of $Mb^{III}F$ resonance Raman spectra. λ_{ex} = 6,000.7 Å. Laser power = 0.37 W. Integration time = 1 sec. Average slitwidth = 4 cm^{-1}. Heme concentration ~0.18 mM. 0.2 M Hepes, 0.2 M. Na_2SO_4, 0.1 M KF, 1.0 mM EDTA.

features except for the peak and trough at 461 and 399 cm^{-1}, respectively. No changes in frequency (±0.5 cm^{-1}) or intensities (±5%) occur for any of the porphyrin macrocyclic vibrational modes. Similarly the three $Hb^{III}F$ pH difference spectra (Figure 6) show large difference features at 407 and 468 cm^{-1} but few spectral differences above 600 cm^{-1} except for a change in the relative intensity of the 760-cm^{-1} heme macrocyclic Raman peak.

Measurements of UV and visible optical absorption spectra revealed pH dependent changes in both $Mb^{III}F$ and $Hb^{III}F$, which are not a result of changes in the extent of fluoride binding (manuscript in preparation). The pH-dependent changes in optical absorption monitored at 540 nm in $Mb^{III}F$ and $Hb^{III}F$, as well as the intensity changes for the 461- and 468-cm^{-1} peaks of $Mb^{III}F$ and $Hb^{III}F$ are plotted in Figure 7. Figure 7 also shows selected titration curves calculated from the Henderson-Hasselbalch equation for various pK values. Both the absorption and resonance Raman $Mb^{III}F$ and $Hb^{III}F$ titration data appear consistent with one proton titrations of some species with an apparent pK = 5.4 ±0.2 for $Mb^{III}F$ and pK = 5.0 ±0.1 for $Hb^{III}F$. The absorption and resonance Raman spectral changes at alkaline pH result from the competitive binding of OH^-.

Discussion and Conclusions

Differences Between Isolated α and β Subunits and Tetrameric $Hb^{III}F$

The resonance Raman difference spectrum in Figure 1 indicates that the Fe—F stretching vibrations at 471 and 444 cm^{-1} in the β subunits rigidly

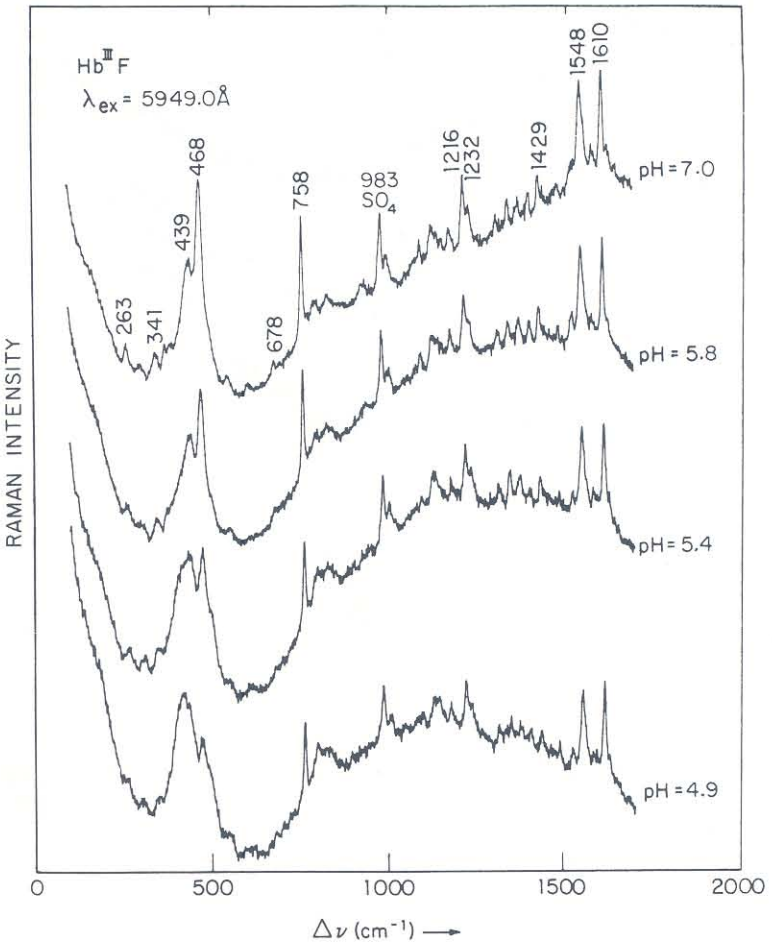


Figure 4. pH dependence of resonance Raman spectra of $Hb^{III}F$. λ_{ex} = 5,949.0 Å. Power = 0.25 W. Integration time = 1 sec. Average slitwidth = 4 cm^{-1}. Heme concentration = 0.31 mM. 0.04 M Na_2SO_4, 0.08 M KF. pH = 7 sample: 0.041 M Hepes. pH = 5.8 sample: 0.04 M Na_2PO_4. pH = 5.4 and 4.9 samples: 0.04 M sodium acetate.

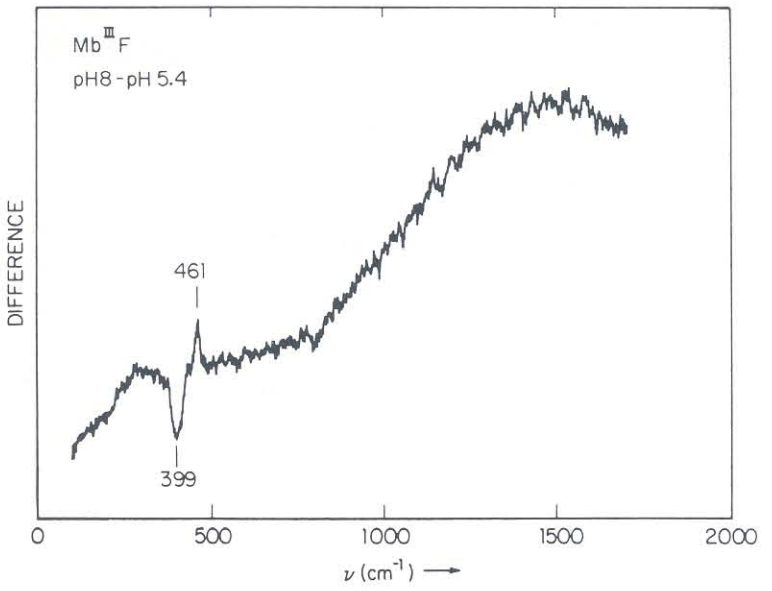


Figure 5. pH difference $Mb^{III}F$ Raman spectrum pH between 8 and 5.4. Individual spectra shown in Figure 3.

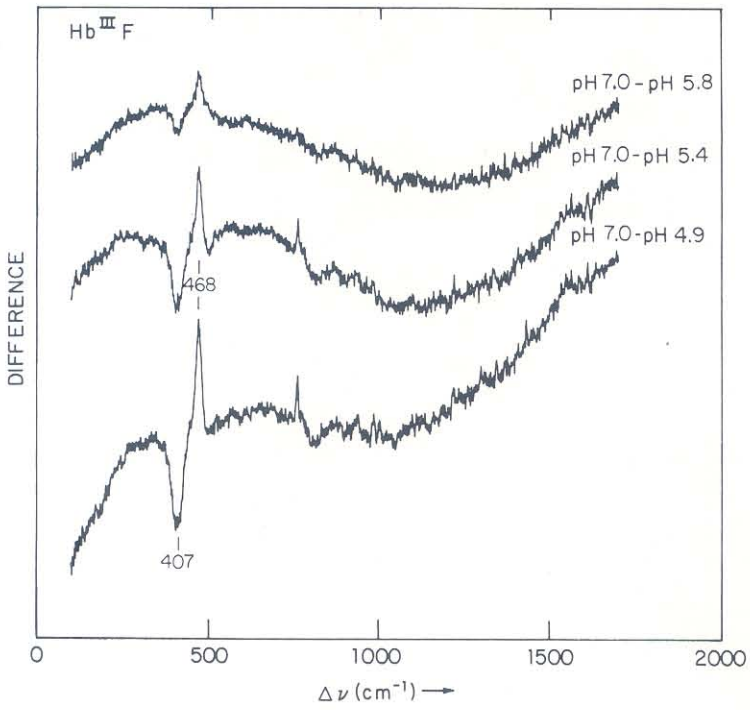
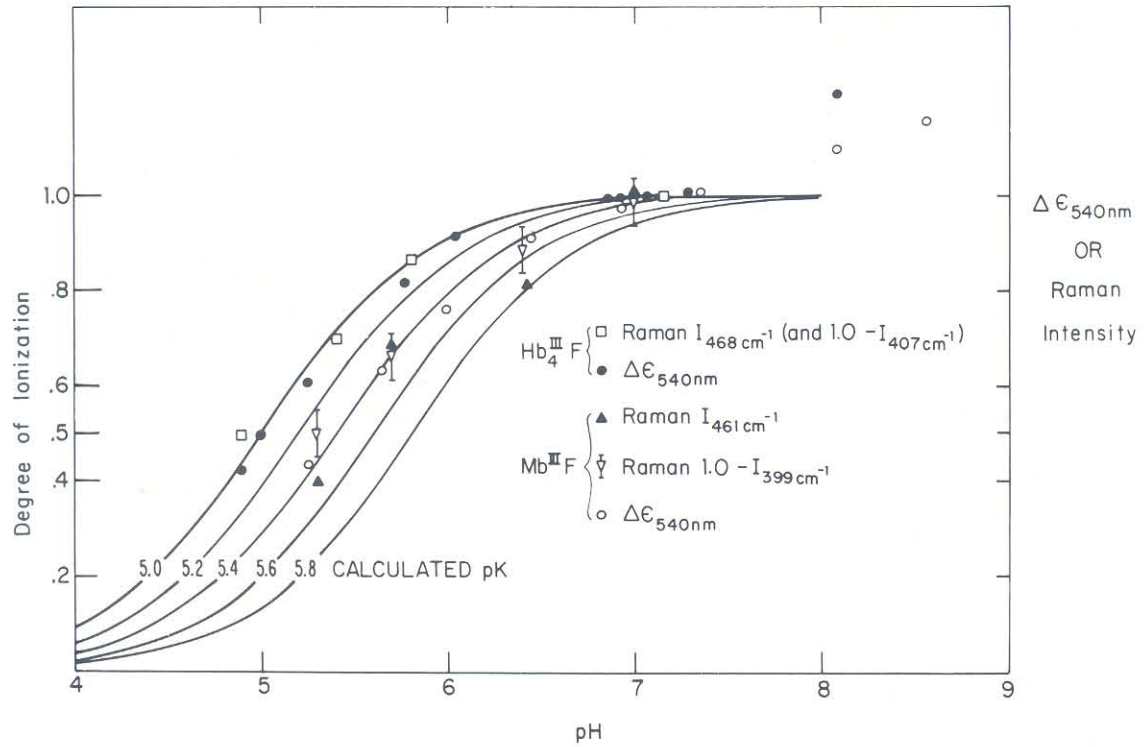


Figure 6. pH difference Hb^{III}F Raman spectra from Figure 4 data.

Figure 7. pH dependence of the absorption spectra monitored at 540 nm and the intensity of the 461- and 399-cm^{-1} Fe—F stretching Raman peaks of Mb^{III}F and the 468- and 407-cm^{-1} Fe—F stretching Raman peaks of Hb^{III}F. Also shown are representative pH titration curves calculated from the Henderson-Hasselbalch equation.



shift 5 cm^{-1} to lower frequency in the α subunits. Associated smaller shifts in the opposite direction to higher frequency occur for the heme macrocyclic modes with the β subunit peak at 1609 cm^{-1} shifting 1.5 cm^{-1} to higher frequency in the α subunits. Because the F^- binding is ionic and the Fe—F vibrations are diatomic stretches, the shifts in the Fe—F stretching vibrations can be related to changes in the Fe—F bond lengths, r by

$$\frac{k_1}{k_2} = \left(\frac{\nu_1}{\nu_2}\right)^2 = \left(\frac{r_2}{r_1}\right)^3$$

where k is the force constant for the Fe—F stretch and ν is the observed stretching frequency (Asher and Schuster, 1979).

Assuming values of 590 cm^{-1} for 5-coordinated nonprotein bound Fe^{III} porphyrin fluoride (Fe^{III}PORF) Fe—F stretching frequencies (see Asher and Schuster, 1979, and references therein) and Fe—F bond lengths of 1.97 Å (the sum of the ionic radii of iron and fluoride), the calculated α and β subunit iron fluoride bond lengths are 2.31 and 2.29 Å, respectively. An apparent elongation of 0.02 Å occurs for the Fe—F bond in the α subunits compared to the β subunits.

We presume that the ~0.3-Å Fe—F bond elongation in protein bound hemes compared to Fe^{III}PORF results from constraints on the heme iron preventing the iron from moving toward the distal side of the heme plane toward the F^- ligand. Since the fluoride is in van der Waal's contact with the nonbonding pyrrole nitrogen orbitals, in 6-coordinated hemes any movement of the iron from the heme plane results in a proportional change in the already strained Fe—F bond (Asher and Schuster, 1979). The data suggest that the iron atoms in the α subunits lie $\gtrsim$ 0.02 Å farther to the proximal side of the heme than in the β subunits. Similarly, the $Mb^{III}F$ iron is calculated to lie $\geq$ 0.05 Å further to the proximal side of the heme plane than in the β subunits.

The strain energy present in the Fe—F bond, due to its elongation from that in a model Fe^{III}PORF where the iron is displaced on the distal side toward the F^- anion can be estimated from

$$E = \frac{(n-1)e^2(r_H - r_o)^2}{4r_o r_H^2},$$

where e is the electronic charge, $n = 10.7$ is the repulsive interaction distance dependence parameter derived from model compounds (Asher and Schuster, 1979). r_H is the Fe—F bond length in the particular heme protein examined and r_o is the Fe—F bond length in a 5-coordinated Fe^{III}PORF. The strain energies calculated in the α and β subunit Fe—F bonds compared to 5-coordinated Fe^{III}PORF model compounds are 8.8 and 8.0 kcal/mole, respectively.

The 1.5-cm^{-1} shift observed for the ~1,610-cm^{-1} Raman peak between the α and β subunits indicates a larger heme core size for the β subunits compared to the α subunits. From numerous empirical studies (Spaulding et al., 1975; Huong and Pommier, 1977; Lanir et al., 1979; Spiro et al., 1979) correlating the peak positions of the ~1,550–1,650-cm^{-1} Raman peaks and the distances $R_{Ct\text{-}N}$ between the center of the heme to the pyrrole nitrogen there appears to be a direct linear correlation between the frequency of the 1,600–1,650-cm^{-1} Raman peak (band V) and the $R_{Ct\text{-}N}$ distance. The relationship discussed by Huong and Pommier (1977) can be used for calculating $R_{Ct\text{-}N}$ for the α and β subunits; $R_{Ct\text{-}N} = 2.068$ Å for the α subunits and $R_{Ct\text{-}N} = 2.073$ Å for the β subunits. The linear correlation between the band V frequencies and the $R_{Ct\text{-}N}$ distances for different metalloporphyrins with different metals and different heme substituents shows scattering with typical deviations larger than the differences we calculate; however, much of the scatter must arise from the different central metals and porphyrin ring substituents, which influence the band V frequencies. Although the exact $R_{Ct\text{-}N}$ distance may differ by as much as 0.01 Å from what we calculate, it is reasonable to expect that for identical hemes the $R_{Ct\text{-}N}\alpha$ and β subunit distance differences calculated should be significant.

The correlation between an *increased* Fe—F bond length and a *decreased* heme core size for the hemoglobin α subunits compared to the β subunits could simply result from an increased ~0.02-Å displacement of the iron to the proximal side of the heme plane. This displacement would elongate the Fe—F bond since the fluoride cannot follow the iron due to contacts with the pyrrole nitrogen orbitals. As the iron moves out of the heme plane the heme core contracts. This contraction is constrained to some degree by steric interactions between the pyrrole nitrogen orbitals and the bound fluoride anion.

An unstrained heme has an $R_{Ct\text{-}N} = 2.01$ Å (Hoard, 1971). Presumably, the heme core of the α and β subunits expands to 2.068 and 2.073 Å, respectively, to accommodate the high-spin iron and its associated ligands. This expansion results in heme macrocycle strain energy, which is

greater for the β subunits than for the α subunits, due to the larger $R_{Ct\text{-}N}$ β subunit distance. Using the $R_{Ct\text{-}N}$ heme bond length and angle dependence proposed by Asher and Schuster (1979) and using a Hookes spring model and force constants derived from normal mode calculations (Asher and Schuster, 1979; Abe et al., 1978), one can estimate the heme strain energy stored from the $R_{Ct\text{-}N}$ distance. Asher and Schuster's expressions result in heme core strain energies of 13.9 and 14.7 kcal for the α and β subunits, respectively, compared to an unstrained heme. It is interesting that the heme core strain energy difference between the α and β subunits is essentially canceled by the strain energy difference present in the Fe—F bonds. Thus, although the α and β subunit hemes are geometrically different, the total strain energy present appears to be identical.

The difference spectrum between tetrameric $Hb^{III}F$ and the isolated subunits indicates that less than a 1-cm^{-1} shift occurs for the Fe—F stretching vibrations upon subunit association into tetrameric $Hb^{III}F$. No shifts are observable for any of the heme macrocyclic vibrational modes. These data suggest that any changes in heme conformation or axial ligand bonding between the isolated α and β subunits and the tetramer due to the unique intersubunit contacts in tetrameric $Hb^{III}F$ are quite small; movements of the iron from the heme plane must be smaller than 0.004 Å. However, it should be noted that the isolated α and β subunit species probably do not exist as monomers in solution at 0.1 mM concentrations we have used for our measurements, but they are associated into dimers and tetramers (Valdez and Ackers, 1977, 1978; Hensley et al., 1975; Ogawa et al., 1972; Bucci et al., 1965; Benesch et al., 1964). Thus, we conclude that the unique α–β subunit contacts in the tetramer do not lead to large changes in heme structure from that found in the isolated chain samples at the concentrations we have used.

The results are in agreement with the suggestions by Perutz et al. (1974 b,c) indicating that no differences in heme geometry occur between the isolated subunits and those hemoglobin tetramers existing in the R conformation. $Hb^{III}F$ is thought to exist in the R conformation in solution (Perutz et al., 1974 a,b). However, Ogawa et al. (1972) observed nmr spectral changes upon $Hb^{III}CN$ subunit association. The presence of these changes would not be detected in the present study since they occur in protein residues not directly bound to the heme.

pH Dependence of $Mb^{III}F$ and $Hb^{III}F$ Spectra

The pH dependence of the absorption and resonance Raman spectra of $Mb^{III}F$ and $Hb^{III}F$ solutions indicate changes in iron coordination at pH values away from neutrality. At high pH the resonance Raman spectra (Figure 3) of $Mb^{III}F$ display a shoulder at ~490cm^{-1}, indicative of the presense of $Mb^{III}OH$ with its characteristic 490-cm^{-1} Fe—O stretching frequency (Asher and Schuster, 1979). No changes are observed in the 1,500–1,700-cm^{-1} Raman spin state region with excitation at ~6,000 Å, because this excitation wavelength enhances only the high-spin $Mb^{III}OH$ species; the high-spin Raman peaks of $Mb^{III}OH$ occur at essentially the same frequencies as do the 1,546- and 1,609-cm^{-1} peaks of $Mb^{III}F$. The presence of an equilibrium between $Mb^{III}OH$ and $Mb^{III}F$ at high pH was noted earlier by nmr and absorption measurements in horse Mb^{III} and human $Hb^{III}F$. These measurements indicate an apparent pK value of ca~10 for equal mixtures of $Mb^{III}F$ and $Mb^{III}OH$ in horse $Mb^{III}F$ (Theorell and Ehrenberg, 1951; Fabray and Eisenstadt, 1974) and a pK_{app} ~ 8.5 in human $Hb^{III}F$ (Gupta, 1975).

Close to neutrality two Fe—F stretching vibrations are observed at 461 and 422 cm^{-1} for $Mb^{III}F$ and at 468 and 439 cm^{-1} for $Hb^{III}F$. The relative intensities of the two Fe—F stretching Raman peaks in each protein depend on the excitation wavelength (Asher et al., 1977); the 461- and 468-cm^{-1} peaks result from diatomic Fe—F stretches, whereas the 422- and 439-cm^{-1} peaks were interpreted as resulting from FeF vibrations lowered in frequency due to hydrogen bonding of the F^- with a water molecule present in some of the heme cavities (Asher and Schuster, 1979; Asher et al., 1977; Deatherage et al., 1976).

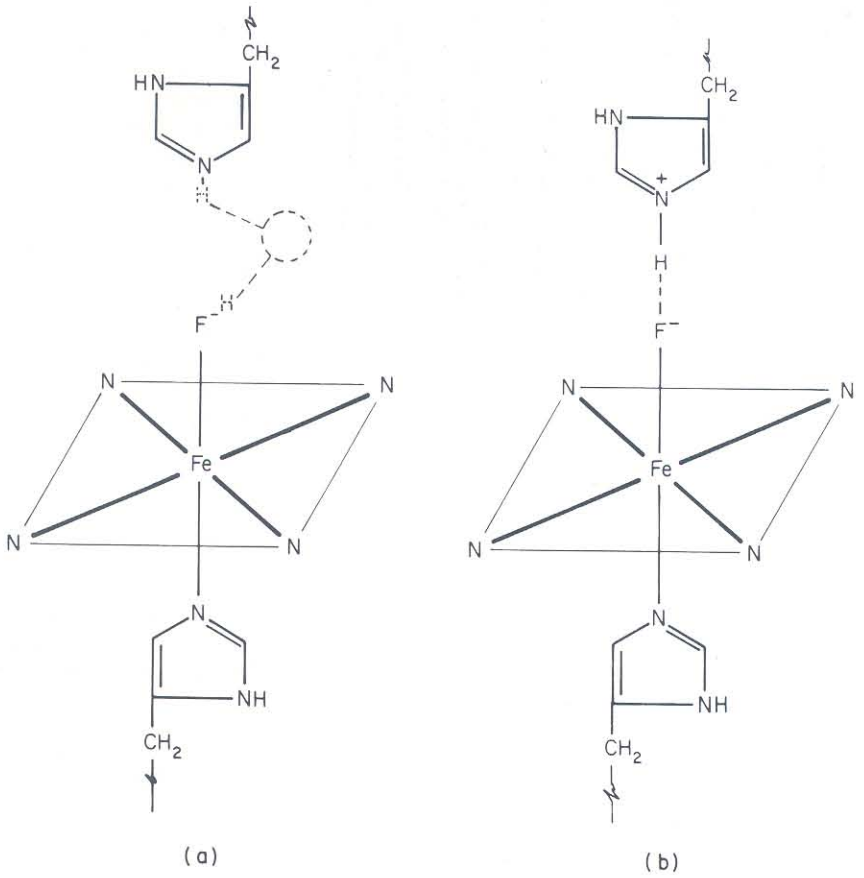
The decrease in intensity of the 461-cm^{-1} ($Mb^{III}F$) and 468-cm^{-1} ($Hb^{III}F$) Fe—F stretches in conjunction with the appearance of 399-cm^{-1} ($Mb^{III}F$) and 407-cm^{-1} ($Hb^{III}F$) peaks at low pH values indicate changes in iron coordination from that at pH = 7. From the difference spectra shown in Figures 5 and 6, which indicate that no shifts occur in any heme macrocyclic vibrational modes, it can be concluded that the heme structural changes are limited to the immediate vicinity of the heme iron. The changes in the axial ligand Raman spectral region can be accounted for either by a perturbation of the Fe—F bonds by some intermolecular interaction between the ligated F^- and the protein, or by a substitution of the F^- ligand by another species in the heme crevice.

Exchange of the F^- ligand is unlikely because the iron remains high-spin (no changes occur in the spin state Raman peaks at 1,546 and 1,609 cm^{-1}) and the absorption spectral changes are small and not characteristic of $\pm F^-$ difference spectra. Exchange of the F^- anion by water can be neglected because the characteristic high-spin aquo complex Raman peak at 1,615 cm^{-1} is not seen and because the 399-cm^{-1} peak is not observed in Mb^{III} H_2O upon excitation between 5,800 and 6,300 Å (manuscript in preparation). Furthermore, the dissociation constant K_L for the reaction $H_2O + Mb^{III}F \rightleftharpoons Mb^{III}H_2O + F^-$ decreases with sample pH indicating increased F^- binding (Theorell and Ehrenberg, 1951; Beetlestone and Irvine, 1969).

The shifts in frequency of the Fe—F stretching vibrations at ~60 cm^{-1} to lower frequency in both $Mb^{III}F$ and $Hb^{III}F$ at low pH presumably are tied to conformational or structural changes in the proteins. We propose that the N_ε of the distal histidine becomes protonated at low pH and hydrogen bonds to the F^- anion, thereby lowering the Fe—F frequency.

This assignment is reasonable since the estimated pK from the data shown in Figure 7 is ~5.0 ±0.1 for $Hb^{III}F$ and 5.4 ±0.2 for $Mb^{III}F$. Our assignments for the Raman doublet and the pH-dependent spectral shifts are shown in Figure 8. Figure 8a indicates the partial occupancy of water in some of the heme crevices at neutral pH

Figure 8. Heme geometry of $Hb^{III}F$ and $Mb^{III}F$. (**A**) pH = 7.0: The fluoride anion is bound to the heme iron. About half of the heme cavities contain a water molecule hydrogen-bonded to the fluoride anion. This partial H_2O occupancy is indicated by the dashed H_2O moiety. The non-hydrogen-bonded FE–F stretching vibration occurs at 461 (468) cm^{-1} in $Mb^{III}F$ ($Hb^{III}F$), whereas the water-hydrogen-bonded FE–F stretching vibration occurs at 423 (439) cm^{-1} in $Mb^{III}F$ ($Hb^{III}F$). (**B**) pH < 6.0: The distal histidine protonates and hydrogen bonds directly to the F^- lowering the Fe–F stretching frequency to 399 (407) cm^{-1} in $Mb^{III}F$ ($Hb^{III}F$).



by indicating the H_2O as dashed. We presume that this water molecule bridges the distal histidine and the fluoride ligand. At low pH values the N_ϵ of the distal histidine protonates and hydrogen bonds directly to the F^- (Figure 8b).

Fabry and Eisenstadt (1974) in nmr studies of horse $Mb^{III}F$ also observed a titration of a group in the heme crevice that changes the optical and proton relaxation behavior of $Mb^{III}F$ solutions. However, in their titration they observed a pK of ~6.0 that they ascribe to protonation of the proximal histidine. This mechanism appears unlikely because protonation of the proximal histidine must result in an increased positive charge at the iron. Since the Fe—F is ionically bound, the force constant would *increase* linearly with charge at the iron, rather than decrease as is observed.

Our conclusion of distal histidine ligation is consistent with extensive literature on the pH dependence of ^{13}C, ^{15}N, and proton nmr spectra of various Mb derivatives. For example, the ^{13}C nmr studies of horse $Mb^{III}CN$ indicate a pK value of 5.3 for either the proximal or distal histidine (Wilbur and Allerhand, 1977), whereas ^{13}CO nmr studies of sperm whale and horse $Mb^{II}CO$ show an apparent pK between 5 and 6 for the ^{13}CO nmr shifts (Moon et al., 1977). Proton nmr studies of sperm whale $Mb^{II}O_2$ indicate distal histidine protonation with a pK = 5.7 (Ohms et al., 1979). In addition, temperature jump measurements (Goldsack et al., 1966) and absorption measurements (Hyashi et al., 1976) of sperm whale Mb^{III}-imidazole and $Mb^{II}CO$ also conclude that the distal histidine hydrogen bonds to the sixth ligand with a pK = 5.7. In addition, epr studies of sperm whale cobalt MbO_2 indicate a pK value of 5.3 for the distal histidine and note that no pH dependence of the epr spectrum occurs in glycera cobalt MbO_2 because of the absence of a distal histidine (Saito, 1977). Our observed pK values for distal histidine protonation and hydrogen bonding are also consistent with ^{15}N nmr studies (Morishima and Inubushi, 1978) of sperm whale $Mb^{III}CN$.

The pK of the distal histidine should be dependent on its molecular environment and the distance that it is constrained by the globin to lie from the fluoride ligand. Protein structural alterations on the distal side of the heme could result in shifts in the distal histidine pK. Our previous studies (Asher et al., 1977) indicate that less than a 0.005-Å change occurs in the iron out-of-heme plane distance upon conversion of $Hb^{III}F$ from the R to the T conformation with inositol hexaphosphate. We have examined the effect of inositol hexaphosphate on the distal histidine pK value using resonance Raman and absorption spectroscopy. Our initial results indicate that any shift in the pK value is less than 0.2 pH units (manuscript in preparation).

Summary

Resonance Raman excitation within hemoglobin and myoglobin charge transfer bands results in enhancement of iron-axial ligand vibrational modes. The frequencies and intensities of the axial ligand vibrations depend upon the detailed heme geometry, iron-out-of-heme-plane distance, and intermolecular interactions between the ligand and the globin. Because the Fe—X (X = F^-, OH^-, N_3^-) vibrations in methemoglobin (Hb^{III}) and metmyoglobin (Mb^{III}) derivatives are uncoupled from any heme macrocyclic vibrational modes, a simple diatomic molecular model can be used to estimate differential strain energies present in the Fe—X bonds. We have examined differences in heme geometry between human $Hb^{III}F$, its isolated α and β subunits, and $Mb^{III}F$. Different iron-out-of-plane distances occur in the isolated α and β subunits. A comparison of the isolated subunits with the $Hb^{III}F$ tetramer indicates that any changes in heme geometry due to the unique α and β subunit contacts present in $Hb^{III}F$ are small. We estimate that the iron is displaced 0.02 Å further from the heme plane in $Hb^{III}F$ α subunits and 0.05 Å further in $Mb^{III}F$ than in $Hb^{III}F$ β subunits. We observe pH dependent changes in the resonance Raman spectra of $Hb^{III}F$ and $Mb^{III}F$, which result from protonation and hydrogen bonding of the distal histidine to the F^- ligand at acid pH. Subtle changes in globin geometry on the distal side of the heme plane can be monitored by examining the pK of the distal histidine and its effect on the Fe—F bond.

We would like to thank Mary Adams for preparing the hemoglobin subunit samples and purifying the myoglobin. We would also like to acknowledge support for this research from the National Institutes of Health (Grants NIH GM-24081-01 to P. S. Pershan and HL-24644 to T. M. Schuster) and the National Science Foundation (Grant PCM 7903074 to T. M. Schuster).

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