Differences in Iron–Fluoride Bonding between the Isolated Subunits of Human Methemoglobin Fluoride and Sperm Whale Metmyoglobin Fluoride As Measured by Resonance Raman Spectroscopy†

Sanford A. Asher* and Todd M. Schuster

ABSTRACT: The heme geometries of the isolated α and β subunits of human methemoglobin fluoride ($Hb^{III}F$) and sperm whale metmyoglobin fluoride ($Mb^{III}F$) have been examined by exciting their Raman spectra within their ca. 6000-Å charge-transfer absorption bands. The Fe–F stretching vibration at 471 cm^{-1} in the β subunits shifts to 466 cm^{-1} in the α subunits and to 461 cm^{-1} in $Mb^{III}F$. The Fe–F bond is estimated to elongate by 0.02 Å in the α subunits and 0.03 Å in $Mb^{III}F$ compared with that in the β subunits. This bond elongation is interpreted to result from an increased iron displacement toward the proximal histidine side of the heme in the series $Mb^{III}F > \alpha > \beta$. A comparison of the isolated subunit spectra with that of tetrameric $Hb^{III}F$ indicates little change occurs in isolated subunit heme geometry upon association into tetrameric $Hb^{III}F$. A correlation is found between the λ_{max} of the 6000-Å charge-transfer absorption band and the Fe–F bond length. Elongation of the Fe–F bond is associated with a shift of the absorption spectral maximum to a longer wavelength. However, the absorption spectral shift induced by the inositol hexaphosphate induced R → T conversion does not result from a change in the Fe–F stretching frequency (±0.5 cm^{-1}). In contrast, frequency shifts are observed for heme macrocyclic vibrational modes. The data are interpreted to indicate that the effect of the R → T conversion in $Hb^{III}F$ is to perturb heme macrocycle conformation without altering the heme out-of-plane iron distance or the Fe–F bond length.

Cooperativity in hemoglobin–ligand binding depends upon the ability of the hemoglobin tetramer to undergo a transition between different quaternary conformations (labeled R and T) which have different ligand affinities [Perutz (1979), Moffat et al. (1979), and references cited therein]. The relevant molecular question is how are the iron–ligand affinities modulated by tertiary and quaternary protein structural changes. Possible mechanisms for decreasing ligand affinities include increases to the proximal histidine side of the out-of-heme-plane iron distances (thereby preventing strong ligand binding), or a blocking of the sixth axial ligand site by amino acid side chains on the distal side of the heme plane. In either case, structural alterations corresponding to shifts in atomic positions of a fraction of an angstrom are expected to be significant energetically.

Any differences in heme geometry between the two quaternary forms of hemoglobin, the α- and β-hemoglobin subunits, and myoglobin must result from different intermolecular interactions between the heme and the surrounding globin. The heme geometry differences occurring between the α and β subunits and myoglobin clearly derive from differences in globin structure. In contrast, changes in the average heme geometry within the α and β subunits between the R and T forms of hemoglobin result from rearrangements of the protein around the heme.

In this study, we have used resonance Raman spectroscopy [for recent reviews, see Warshel (1977) and Yu (1977)] to probe the differences in heme geometry between the iron(III) fluoride derivatives of the isolated α and β subunits ($\alpha^{III}F$, $\beta^{III}F$),[1] the tetramer in both the R and T conformations ($Hb^{III}F$), and myoglobin ($Mb^{III}F$). By excitation within the 6000-Å charge-transfer electronic transition of the heme, both heme macrocycle and axial ligand vibrational modes are enhanced (Asher & Schuster, 1979, 1980; Asher et al., 1977; Asher & Sauer, 1976). By examination of both sets of vibrational modes, any globin perturbations of heme geometry can be assigned either to interactions with the iron atom and its axial ligands or to the heme aromatic macrocycle. Because the fluoride anion is bound ionically and the Fe–F stretching vibration is a diatomic stretch uncoupled from other heme vibrational modes (Asher & Schuster, 1979; Asher et al., 1977), quantitative relationships can be derived between the Fe–F stretching frequency and the Fe–F bond length (Asher & Schuster, 1979). The Fe–F bond length can, in turn, be related to the iron out-of-heme-plane distance.

Experimental Procedures

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 a salt-free lyophilized powder. The major component of human hemoglobin, A_0, was purified as oxyhemoglobin by the method of Williams & 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 & Fronticelli (1965), and the blocked SH groups were regenerated by the method of Geraci et al. (1969). Methemoglobin and

† From the Gordon McKay Laboratory, Division of Applied Sciences, Harvard University, Cambridge, Massachusetts 02138 (S.A.A.), and the Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268 (T.M.S.). *Received September 2, 1980.* This work was supported by National Institutes of Health Grants GM-24081-01 (to P. S. Pershan) and HL-24644 and National Science Foundation Grant PCM 79-03964 (to T.M.S.).

* Correspondence should be addressed to this author at the Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260.

[1] Abbreviations used: α^{III}, isolated methemoglobin α chains; β^{III}, isolated methemoglobin β chains; Mb^{III}, metmyoglobin; Hb^{III}, methemoglobin; $Fe^{III}POR$, ferric porphyrin; R_{Ct-N}, distance between the center of the porphyrin and the pyrrole nitrogens; Fe–N_ϵ, iron–proximal histidine bond; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $InsP_6$, inositol hexaphosphate.

0006-2960/81/0420-1866$01.25/0 © 1981 American Chemical Society

metmyoglobin fluoride samples were prepared by addition of the purified protein to buffer solutions followed by addition of the appropriate amounts 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.

Absorption spectra were obtained on each sample both before and after the Raman spectral measurements by using a Cary 14 (Varian) instrument. The absorption spectra were identical, indicating little or no sample decomposition occurred during the Raman spectral measurement. Absorption spectra and difference spectra were obtained by using a computer-controlled Cary 118 C (Varian) instrument.

The Raman spectra were measured, using a rotating cell to avoid sample decomposition, on a microcomputer-interfaced Raman spectrometer described in detail elsewhere (Asher & Schuster, 1979). The Raman difference spectra were obtained by direct numerical subtraction of the original data. The data used for the difference spectra were obtained within a few hours of one another, and the absence of any shifts for the internal sulfate Raman vibration at 983 cm^{-1} indicates that no significant shift in spectrometer alignment or laser wavelength occurred between the individual Raman measurements. Further, measurements of identical samples at both the beginning and the end of the experimental day result in totally featureless Raman difference spectra.

Differences spectra between $HB^{III}F$ and the sum of the spectra of the isolated subunits were obtained by scaling the sum of the α- and β-subunit spectra such that the intensities of the heme macrocycle Raman peaks were identical with that of tetrameric $Hb^{III}F$. The spectra were then numerically subtracted. The scaling used for all of the difference spectra was within 7% of that expected to account for differences in sample concentration and the number of spectral scans. This suggests that the relative Raman cross sections of the heme macrocycle vibrations are almost identical in the subunits and the tetramer.

Results

Absorption spectral differences occur between the isolated α and β subunits of hemoglobin and myoglobin (Yonetani et al., 1971; George et al., 1964; Antonini & Brunori, 1971). For ferric derivatives, the spectral differences often result from spin-state equilibrium differences where the proportion of the high- and low-spin species differs between the proteins (Yonetani et al., 1971; Iizuka & Kotani, 1969; Beetlestone & George, 1964; George et al., 1964). These spin-state differences indicate the presence of a protein-dependent iron coordination which may be related to the differences observed in the ligand binding affinities between these proteins (Moffat et al., 1979).

Figure 1 shows the absorption spectra of the fluoride derivatives of the isolated α and β subunits of methemoglobin, of the $Hb^{III}F$ tetramer, and of $Mb^{III}F$. Although the absorption spectra of these derivatives are very similar, small shifts are observed for the band maxima. These spectral differences between $Hb^{III}F$ and $Mb^{III}F$ do not result from spin-state differences because each of these fluoride derivatives is essentially 100% high spin (Beetlestone & George, 1964; Perutz et al., 1974c). The charge-transfer bands at ca. 6000 Å occur at 6040, 6060, and 6065 Å for the isolated β subunits, α subunits, and myoglobin, respectively.

The differences between the absorption spectra of these proteins reflect differences in the interactions between the heme and the globin. We have examined heme geometry differences by measuring the resonance Raman spectra of these derivatives with excitation within the 6000-Å charge-transfer absorption

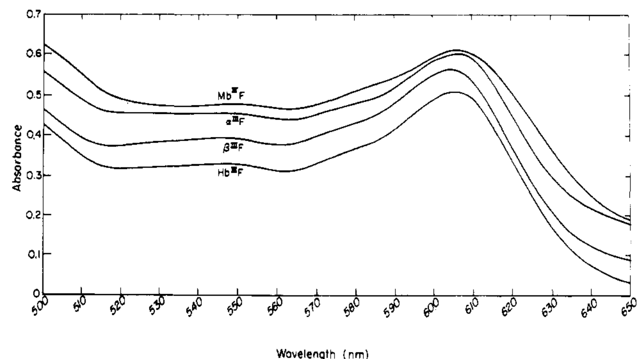

FIGURE 1: Absorption spectra of the fluoride derivatives of methemoglobin, the isolated human methemoglobin α and β subunits, and metmyoglobin fluoride. Path length = 1 cm. Heme concentrations: $Hb^{III}F$ = 63 μM, $\alpha^{III}F$ = 53 μm, $\beta^{III}F$ = 55 μM, $Mb^{III}F$ = 64 μM, 0.1 M KF, 0.05 M Hepes buffer, pH 6.8. The spectra have been vertically shifted to make comparisons easier.

bands (Eaton & Hochstrasser, 1968; Smith & Williams, 1970). Excitation within these electronic transitions enhances both iron–axial ligand vibrational modes, which are sensitive to the heme geometry (Asher et al., 1977; Asher & Schuster, 1979), and heme macrocyclic modes, which monitor the heme electron density and the heme core size (Spaulding et al., 1975; Kitagawa et al., 1976; Huong & Pommier, 1977; Spiro & Burke, 1976; Lanir et al., 1979; Spiro et al., 1979).

Figure 2 shows the resonance Raman spectra of the isolated $\alpha^{III}F$ and $\beta^{III}F$ chains and their difference spectrum while Figure 3 shows the resonance Raman spectra of $Mb^{III}F$ and a difference spectrum between $\alpha^{III}F$ and $Mb^{III}F$. The high-frequency region of the Raman spectra exhibits porphyrin macrocyclic modes at ca. 1550 and 1610 cm^{-1}, indicating a high-spin iron (Spiro & Burke, 1976; Spiro, 1975; Kitagawa et al., 1976). The 983-cm^{-1} peak is contributed by sulfate ion added to each of the samples in the form of Na_2SO_4 as an internal frequency and intensity standard.

The ca. 470- and 440-cm^{-1} peaks in the Raman spectra have been previously assigned to Fe–F diatomic stretching vibrations (Asher & Schuster, 1979; Asher et al., 1977). The 470- and 440-cm^{-1} doublet was assigned to Fe–F stretching vibrations on the basis of the following: (1) the unique appearance of the doublets in the Raman spectra of F^- derivatives; (2) the similarity of the 6000-Å charge-transfer absorption band excitation profiles of the doublets to the excitation profiles of the unequivocably assigned Fe–O stretching vibrations in the hydroxide complexes [the high-spin 6000-Å hydroxide derivative absorption band appears to result from the same electronic transition as does that of the fluoride complexes (Churg et al., 1979)]; (3) the unique frequency shift of the doublet between $Hb^{III}F$ and $Mb^{III}F$; the doublet frequency shift which is in the same direction as that of the Fe–O stretch in the hydroxide complexes (Asher & Schuster, 1979) is the only feature of the Raman spectra of fluoride derivatives to show a large frequency difference between $Mb^{III}F$ and $Hb^{III}F$.

The assignment of the ca. 460–470-cm^{-1} peaks to Fe–F stretches is strongly supported by the recent resonance Raman study of Desbois et al. (1979) in which a 2-cm^{-1} shift of the 461-cm^{-1} $Mb^{III}F$ peak occurs upon isotopic substitution of ^{54}Fe for ^{56}Fe; this is the expected shift for a diatomic Fe–F stretch.

The lower frequency component of the doublet at ca. 440 cm^{-1} in $Hb^{III}F$ and at 420 cm^{-1} in $Mb^{III}F$ was conjectured to result from an Fe–F stretch, lowered in frequency due to hydrogen bonding of the F^- anions to a water molecule present in about half of the heme cavities (Asher et al., 1977; Asher & Schuster, 1979). A heterogeneity within the heme cavities

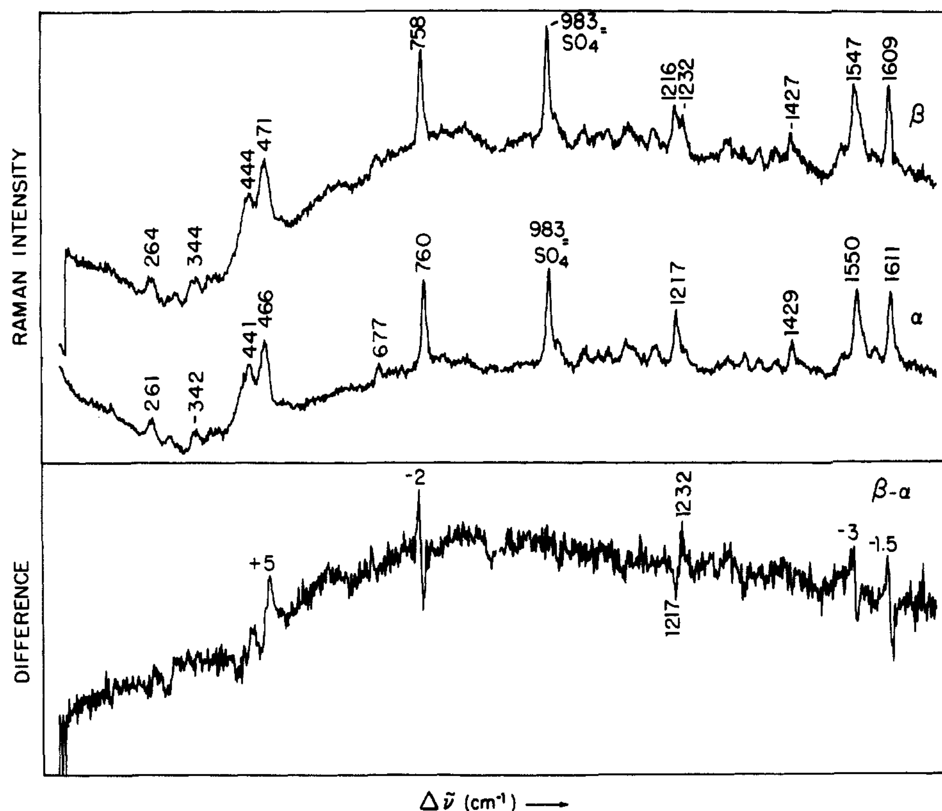

FIGURE 2: Resonance Raman spectra of isolated α and β chains and their difference spectrum. λ_{ex} = 6050.6 Å; average slit width = 4 cm^{-1}; integration time = 1 s; power = 0.37 W. Heme concentration = 0.255 mM, 0.2 M Na_2SO_4, 0.2 M Hepes buffer, pH 7.0, 0.1 M KF, 1.0 mM EDTA. The 983-cm^{-1} peak results from the internal standard SO_4^{2-} vibrational mode.

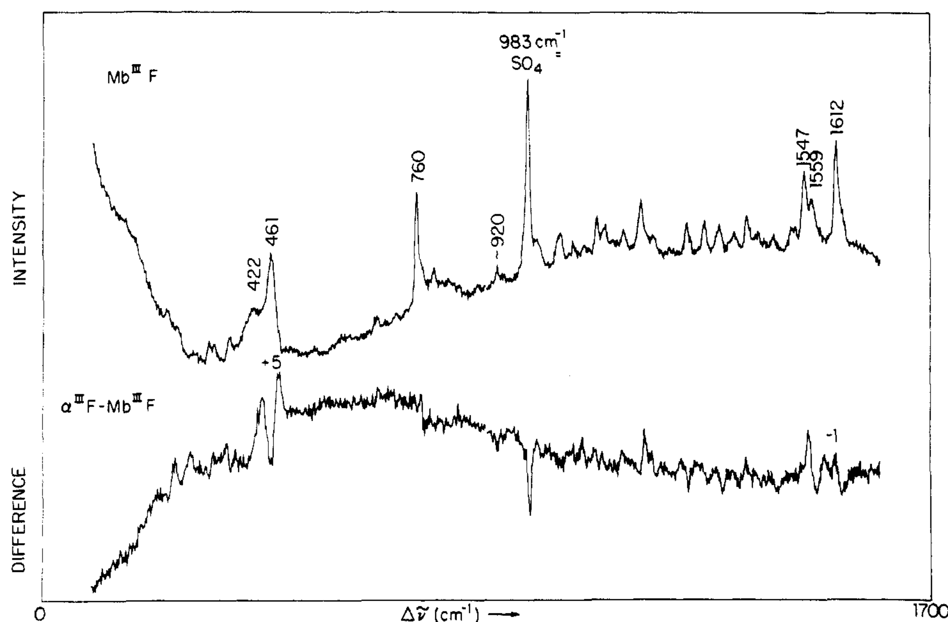

FIGURE 3: Resonance Raman spectrum of Mb^{III}F and the difference spectrum between α^{III}F and Mb^{III}F. α^{III}F sample conditions were as in Figure 2. Mb^{III}F sample conditions: λ_{ex} = 6000.7 Å; laser power = 0.37 W; integration time = 1 s; average slit width = 4 cm^{-1}. Heme concentration ca. 0.18 mM, 0.2 M Hepes, 0.2 M Na_2SO_4, 0.1 M KF, 1.0 mM EDTA.

with hydrogen bonding of some species (possibly water) to the fluoride anion was noted earlier by Deatherage et al. (1976) in the X-ray diffraction studies of Hb^{III}F. Heterogeneity in ligand binding has previously been observed to occur in carbonmonoxy derivatives of myoglobin (Makinen et al., 1979; Fuchsman & Appleby, 1979; McCoy & Caughey, 1970, 1971). Further, Koenig et al. (1980) have recently presented proton NMR relaxation data indicating the presence of an exchangeable water molecule near the heme iron in Mb^{III}F [however, see Gupta & Mildvan (1975)].

The difference spectrum between the isolated α and β subunits (Figure 2) indicates frequency shifts for many of the Raman peaks. The ca. 760-, 1550-, and 1610-cm^{-1} porphyrin macrocyclic vibrational modes in α^{III}F are higher in frequency than the corresponding ones in β^{III}F. 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. 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. The difference spectrum between α^{III}F and Mb^{III}F (Figure 3) indicates that

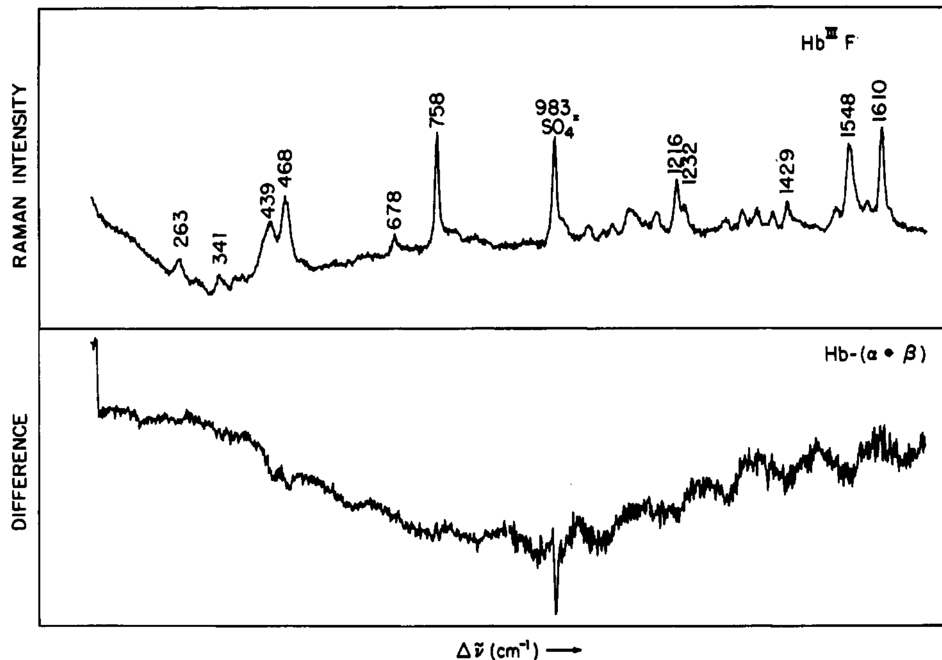

FIGURE 4: Resonance Raman spectrum of $Hb^{III}F$ and the difference spectrum between tetrameric $Hb^{III}F$ and the sum of the spectra of $\alpha^{III}F$ and $\beta^{III}F$. $Hb^{III}F$ heme concentration = 0.261 mM. Isolated subunit conditions were as in Figure 2. The 983-cm^{-1} trough in the difference spectrum results from an increased SO_4^{2-} concentration in the isolated subunits compared to the tetramer sample.

the Fe–F stretch occurs at 461 cm^{-1} in $Mb^{III}F$, 5 cm^{-1} to lower frequency from that of the α subunits and 10 cm^{-1} to lower frequency from that of the β subunits. The 1612-cm^{-1} peak in $Mb^{III}F$ is ca. 1 cm^{-1} higher in frequency than that of the α subunits and 2–3 cm^{-1} higher in frequency than that of the β subunits. It is difficult to estimate the shift occurring for the 1550-cm^{-1} peak due to the overlap of a peak at ca. 1560 cm^{-1} in $Mb^{III}F$.

If Figure 4, we compared the heme geometry within the isolated α and β subunits with that in tetrameric $Hb^{III}F$. The 468-cm^{-1} Fe–F stretching vibration in tetrameric $Hb^{III}F$ is 3 cm^{-1} lower than that reported by Asher et al. (1977). However, this difference is within the estimated accuracy of our previous measurements by using different instrumentation.

The lower part of Figure 4 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 at ca. 1550 and 1610 cm^{-1} in the spectral sum of the isolated chains such that they were identical with that in the tetramer. The scaling factor was within 7% 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 4) 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 that of 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 of the α and β Fe–F stretching vibrations from that of the tetramer. The observed Fe–F stretching frequencies in $Hb^{III}F$ (which are the average of the Fe–F stretching frequencies of the α and β subunits) are decreased by ca. 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 (Figure 2), the data cannot unequivocably resolve whether this α- and

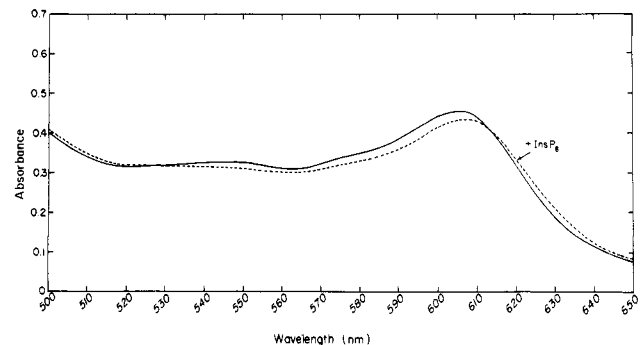

FIGURE 5: Absorption spectra of $Hb^{III}F$ before and after $InsP_6$ addition. Sample conditions were as in Figure 6. Path length 0.2 cm.

β-chain difference is preserved in the tetramer because the Fe–F stretching frequencies in the tetramer chains could have shifted in equal and opposite directions relative to that in the isolated chains. However, it is clear that the unique intersubunit contacts in the tetramer result 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.

The absorption spectra in Figure 1 indicate a shift of the ca. 6000-Å absorption band to a longer wavelength in the series $Mb^{III}F$ > α subunits > β subunits. The Raman spectra (Figures 2 and 3) indicate that the force constant of the Fe–F stretch increases in the series $Mb^{III}F$ < α subunits < β subunits. Thus, a red shift in the absorption spectrum correlates in these derivatives with a decrease in the force constant of the Fe–F bond.

Binding of inositol hexaphosphate ($InsP_6$) to $Hb^{III}F$ causes a shift in the λ_{max} of the 6000-Å absorption band to a longer wavelength as illustrated in Figure 5. The molecular origin of this spectral shift is not known but presumably results from the well-characterized (Perutz et al., 1974a,b, 1978; Fermi & Perutz, 1977) R → T quaternary structural conformational change caused by $InsP_6$ binding; the absorption spectral change is clearly not a result of a spin-state change since the fluoride derivatives are all essentially 100% high spin (Beetlestone & George, 1964; Perutz et al., 1974c). Previously, with less

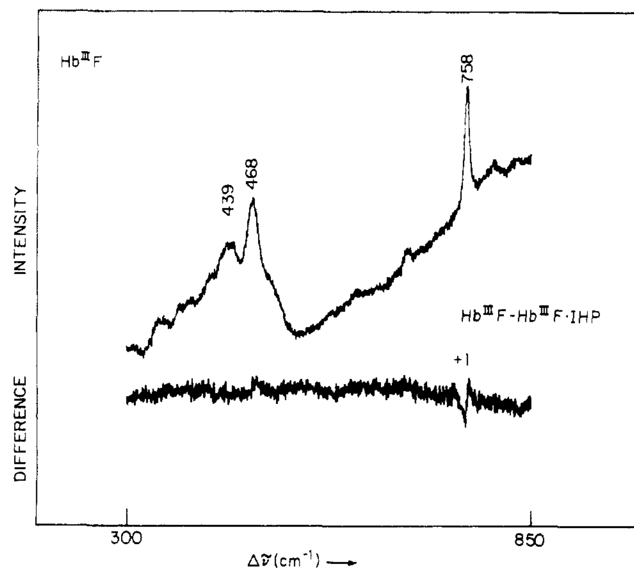

FIGURE 6: Resonance Raman spectrum of Hb^{III}F and the difference spectrum after InsP$_6$ addition. Heme concentration = ca. 0.3 mM; InsP$_6$ concentration = 7.5 mM, pH 6.86, 0.95 M Hepes, 0.1 M KF. λ_{ex} = 6000.0 Å; laser power = 0.3 W; integration time = 1 s; average slit width = 4 cm^{-1}.

sensitive instrumentation (Asher et al., 1977), we examined the effect of InsP$_6$ on the Fe–F stretching frequency and found that any frequency shift was limited to less than ±3 cm^{-1}. We interpreted this result as indicating that less than a 0.01-Å movement of the iron atom from the heme plane occurred due to the InsP$_6$-induced R → T conversion. More recently, Rousseau et al. (1980) used a Raman difference spectral technique to examine the effect of InsP$_6$ upon Hb^{III}F heme macrocyclic vibrational modes and found that a ca. 0.6-cm^{-1} frequency shift occurs in certain heme macrocycle vibrational modes. We have reexamined the effect of InsP$_6$ on the force constant of the Fe–F bond by using our current instrumentation.

Figure 6 shows that Raman difference spectrum for Hb^{III}F in the presence and absence of InsP$_6$. The data indicate that less than a 0.5-cm^{-1} shift occurs for the Fe–F stretching vibration although the heme macrocyclic vibrational mode at 760 cm^{-1} does show a small shift of ca. 1 cm^{-1}. In contrast to the correlation observed between the Fe–F stretching frequency and the λ_{max} of the 6000-Å charge-transfer absorption band in the Hb^{III}F isolated α subunits, isolated β subunits, and Mb^{III}F, the substantial absorption spectral shift accompanying InsP$_6$-induced R → T conversion in Hb^{III}F is not correlated with any observable shift in the frequency of the Fe–F stretch (±0.5 cm^{-1}). This result and the observations of frequency shifts for heme macrocyclic vibrational modes [this study and Rousseau et al. (1980)] suggest that the changes in heme–globin interactions which result from the R → T conversion and which lead to absorption spectral shifts are localized in the heme ring and not at the iron atom.

Discussion

Differences between Isolated α and β Subunits, Mb^{III}F, and Tetrameric Hb^{III}F. The resonance Raman difference spectra in Figures 2 and 3 indicate that the Fe–F stretching vibrations at 471 and 444 cm^{-1} in the β subunits rigidly shift 5 cm^{-1} to lower frequency in the α subunits and shift another 5 cm^{-1} lower in frequency in Mb^{III}F. 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} shifted 1.5 cm^{-1} to higher frequency in the α subunits and an additional ca. 1 cm^{-1} in Mb^{III}F. Because the F$^-$ bonding 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 & Schuster, 1979).

With the assumption of values of 590 cm^{-1} for 5-coordinate non-protein-bound iron(III) porphyrin fluoride (Fe^{III}PORF) Fe–F stretching frequencies [see Asher & Schuster (1979) and references cited therein], and Fe–F bond lengths of 1.97 Å (the sum of the ionic radii of iron and fluoride), the calculated Mb^{III}F and α- and β-subunit iron to fluoride bond lengths are 2.32, 2.31, and 2.29 Å, respectively; apparent elongations of 0.03 and 0.02 Å occur for the Fe–F bonds in Mb^{III}F and the α subunits compared to those in the β subunits, respectively.

We presume that the ca. 0.3-Å Fe–F bond elongation in protein-bound hemes compared to Fe^{III}PORF results from globin 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-coordinate hemes any movement of the iron from the heme plane results in a proportional change in the already strained Fe–F bond (Asher & Schuster, 1979).

The 1.5-cm^{-1} difference observed for the ca. 1610-cm^{-1} Raman peak between the isolated α and β subunits indicates an increased heme core size for the β subunits over that of the α subunits. A similar analysis of Mb^{III}F indicates it has a slightly smaller heme core size than the α subunits. From numerous empirical studies (Spaulding et al., 1975; Huong & Pommier, 1977; Lanir et al., 1979; Spiro et al., 1979) correlating the peak positions of the ca. 1550–1650-cm^{-1} Raman peaks and the distance between the center of the heme to the pyrrole nitrogens, R_{Ct-N}, there appears to be a direct linear correlation between the frequency of the 1600–1650-cm^{-1} Raman peak (band V) and the R_{Ct-N} distance. Huong & Pommier's (1977) relationship can be used to calculate R_{Ct-N} = 2.066 Å for Mb^{III}F, R_{Ct-N} = 2.068 Å for the α subunits, and R_{Ct-N} = 2.073 Å for the β subunits. The linear correlation between the band V frequencies and the R_{Ct-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 absolute R_{Ct-N} distances may differ by as much as 0.01 Å from what we calculate, it is reasonable to expect that for identical hemes the relative R_{Ct-N} α- and β-subunit distance difference calculated should be significant.

The suggested correlation between an *increased* Fe–F bond length and a *decreased* heme core size for Mb^{III}F, α^{III}F, and β^{III}F could simply result from variable displacement of the iron to the proximal side of the heme plane. This displacement would be expected to 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.

The decreased Fe–F force constant and increased bond length in Mb^{III}F from that in Hb^{III}F (which is the average of the α and β subunits) may be directly associated with the decreased fluoride binding affinity observed for Mb^{III}F com-

pared to $Hb^{III}F$ (Antonini & Brunori, 1971). From the Raman data, it appears that the different globin constraints present in the $Hb^{III}F$ subunits and $Mb^{III}F$ result in heme geometry differences. Because these geometric differences can be interpreted in terms of bond length changes, it is possible to estimate that strain energy differences due to the changes in the R_{Ct-N} and Fe–F bond lengths calculated for these proteins. These strain energy differences represent changes in the local heme internal energy which may be directly related to experimentally observed differences in ligand binding enthalpies and affinities.

The strain energy present in the Fe–F bond, due to its elongation from that in a model $Fe^{III}PORF$, can be estimated from (Asher & Schuster, 1979)

$$E = \frac{(n-1)e^2(r_H - r_0)^2}{4r_0 r_H^2}$$

where e is the electronic charge, $n = 10.7$ is the repulsive interaction distance dependence parameter derived from model compounds, r_0 is the Fe–F bond length in a 5-coordinate $Fe^{III}PORF$, and r_H is the Fe–F bond length in the heme protein. The strain energies calculated in the α- and β-subunit Fe–F bonds compared to 5-coordinate $Fe^{III}PORF$ model compounds are 8.8 and 8.0 kcal/mol, respectively. The strain energy for $Mb^{III}F$ is 9.3 kcal/mol.

An unstrained heme has an R_{Ct-N} value of 2.01 Å (Hoard, 1971). The heme core of $Mb^{III}F$ and the α and β subunits expands to 2.066, 2.068, and 2.073 Å, respectively, relative to an unstrained heme, presumably to accomodate the high-spin iron and its associated ligands. This expansion results in a larger macrocycle strain energy for the β subunits than for $Mb^{III}F$ or the α subunits, due to the larger β-subunit R_{Ct-N} distance.

The strain energies involved in the R_{Ct-N} expansion in $\alpha^{III}F$ and $\beta^{III}F$, compared to that of an unstrained heme where $R_{Ct-N} = 2.01$ Å, can be estimated from (Asher & Schuster, 1979)

$$\Delta E = 2K(\Delta r_3)^2 + 2H_1 r_2^2(\Delta\phi)^2 + 4H_2 r_1 r_2(\Delta\psi)^2$$

where K is the stretching force constant for an iron–pyrrole–nitrogen bond, H_1 is the bending force constant of the C_α–C_M–C_α linkage, and H_2 is the bending force constant for the N–C_α–C_M link. With the assumption of the values of Abe et al. (1978), $k = 0.7$, $H_1 = 0.79$, and $H_2 = 0.32$ mdyn/Å where Δr_3, $\Delta\phi$, and $\Delta\psi$ are the extensions of the iron–pyrrole–nitrogen bonds and the angle deformations over those in an unstrained heme. ϕ is the angle subtended by the bonds linking the α carbons of two different pyrrole rings to the methine carbon (η_α–C_M–C_α), and ψ is the angle subtended by the pyrrole nitrogen–α-carbon–methine carbon linkage (C_N–C_α–C_M). θ is the half-angle between the bonds connecting the C_α carbons of one pyrrole and its pyrrole nitrogen, r_1 is the bond length between the α carbon and the pyrrole nitrogen, r_2 is the bond length between C_α and the methine carbon, and r_3 is the length of the Fe–N (pyrrole) bond. Correcting for a topographical error in Asher & Schuster (1979) yields

$$\Phi = \pi - 2\cos^{-1}\frac{\{2[R_{Ct-N} + r_1(\cos\theta - \sin\theta)]^2\}^{1/2}}{2r_2}$$

$$\psi = \frac{3\pi}{4} + \theta - \Phi/2$$

These expressions result in heme core strain energies of 13.9 and 14.7 kcal/mol for the α and β subunits, respectively, compared to an unstrained heme. It is interesting to note 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 net strain energy present in the heme core and in the Fe–F bond appears to be identical.

There appears to be a net difference of ca. 0.9 kcal/mol of heme between the average strain energy present in the Fe–F bonds of $Hb^{III}F$ and $Mb^{III}F$. This is close to the experimentally observed 1 kcal/mol of heme difference in the fluoride binding enthalpies between $Mb^{III}F$ and $Hb^{III}F$ (Antonini & Brunori, 1971).

$Hb^{III}F$ Tetramer. The difference spectrum between tetrameric $Hb^{III}F$ and the isolated subunits (Figure 4) 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 α- and β-intersubunit peptide contacts in tetrameric $Hb^{III}F$ are quite small; the average change in the Fe–F bond length is estimated to be less than 0.004 Å, suggesting than any movement of the iron from the heme plane must be of a similar small magnitude. However, it should be noted that the isolated α- and β-subunit species probably do not exist as monomers in solution at the 0.1 mM concentrations we have used for our measurements, but are associated into dimers and tetramers (Valdez & 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 23% decrease in the Raman intensity of the Fe–F stretches relative to the 1550- and 1610-cm^{-1} peaks could indicate a decreased ligand binding affinity for fluoride. However, the resulting presence of the aquomet derivative should give rise to a difference spectral feature in Figure 4 at ca. 1640 cm^{-1}, which is not observed; Raman excitation of aquomethemoglobin and aquometmyoglobin at ca. 6050 Å enhances low spin-state heme derivative Raman peaks at ca. 1640 cm^{-1} (Asher et al., 1981). The Fe–F stretch intensity decrease could also result from shifts in the excitation profiles of the 440–470-cm^{-1} and the 1550- and 1610-cm^{-1} peaks. Because the excitation profiles of these vibrational modes are sharply peaked at different positions within the 6000-Å absorption band (Asher et al., 1977), small shifts in either the absorption spectra or the excitation profiles maxima could lead to significant changes in the relative Raman intensities for the isolated subunits compared to the tetramer.

These results are in agreement with the suggestions by Perutz et al. (1974a,b), indicating that no large 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., 1974b,c).

The Raman data allow us to estimate those strain energies in the heme due to changes in the R_{Ct-N} distances and the iron–axial ligand bond lengths. However, other factors do contribute to the internal energy of the heme such as strains in the iron–N_ϵ (proximal histidine) bond and strain energies due to the 1–3 interactions of the pyrrole nitrogen orbitals with the fluoride anion and with the proximal histidine (Warshel, 1977). The total strain energy can be written

$$E = V_H + V_{Fe-F} + V_{Fe-N} + V_F + V_N + V_P$$

where V_H is the heme macrocyclic strain energy, V_{Fe-F} (V_{Fe-N})

is the strain energy in the Fe–F [Fe–N_ϵ (proximal histidine)] bond, V_F and V_N are the strain energies resulting from 1–3 interactions of the fluoride and proximal histidine with the pyrrole nitrogen orbitals, and V_P is the strain energy within the globin. Changes in globin and heme conformation between different heme derivatives result from the protein and heme conformational structural changes required to minimize E.

The differences in strain energy between the α and β subunits are

$$\Delta E = \Delta V_H + \Delta V_{Fe-F} + \Delta V_{Fe-N} + \Delta V_F + \Delta V_N + \Delta V_F$$

The Raman measurements permit us to estimate ΔV_H and ΔV_{Fe-F}. However, since we have not observed any spectral features characteristic of the Fe–N_ϵ (proximal histidine) linkage, we have no estimate of ΔV_{Fe-N}. We also do not know ΔV_P. We have estimated $\Delta V_H = -\Delta V_{Fe-F}$. ΔV_F and ΔV_{Fe-N} should scale with ΔV_{Fe-F} while ΔV_N may decrease. Thus, at the heme, unless $\Delta V_N > \Delta V_{Fe-F} + \Delta V_{Fe-N}$, $E_\alpha > E_\beta$. Thus, in the fluoride derivatives, the internal heme energy should be larger for the α subunits than for the β subunits. This may suggest that the α subunits will have a smaller affinity for fluoride than do the β subunits. However, in aqueous solution, the fluoride affinity is measured relative to the affinity for water molecules. Thus, the typically measured fluoride affinity also depends on the relative affinities of α^{III} and β^{III} for H_2O.

Absorption Spectral Shifts and Axial Ligand Force Constants. The shift in the λ_{max} of the ca. 6000-Å absorption spectra (Figure 1) to longer wavelengths as the length of the Fe–F bond increases can be rationalized in terms of a coulombic repulsive interaction of the F^- anion with the d electrons of the iron atom. Since the 6000-Å charge-transfer absorption band is x, y polarized (Eaton & Hochstrasser, 1968), the electronic transition involves excitation from the highest occupied orbitals of a_{1u} and a_{2u} symmetry to a molecular orbital with significant d_{xz}, d_{yz} character. A change in the distance of the fluoride from the heme iron perturbs the energy levels of the ground and excited states to first order by a potential term:

$$V' = \sum_{i=1}^{m} \frac{q_e q_F}{|\hat{\mathbf{r}}_i - \hat{\mathbf{R}}|^2}$$

where q_e is the electronic charge, q_F is the charge of the fluoride anion, $\hat{\mathbf{R}}$ is a vector designating the position of F^- relative to the heme iron, and $\mathbf{r}_i$ is a vector designating the position of the ith electron in the heme iron complex.

A change in $\hat{\mathbf{R}}$ affects both the ground- and excited-state energies. To lowest order, the electronic transition will be shifted by an amount

$$\Delta E = \langle e|V'|e\rangle - \langle g|V'|g\rangle$$

Since the electrons in the iron d orbitals are closest to the F^- ligand, they will be more perturbed by changes in $\mathbf{R}$ than will porphyrin π electrons. Thus, the excited state reached by a $\pi \rightarrow$ d charge-transfer transition will be shifted toward a longer wavelength as the Fe–F bond lengthens more than will a $\pi \rightarrow \pi^*$ transition. Any neutralization of fluoride anion charge would also be expected to lead to a red shift in the absorption spectrum. This is precisely what occurs when the distal histidine protonates at low pH in $Hb^{III}F$ and $Mb^{III}F$ and hydrogen bonds to the fluoride ligand (Asher & Schuster, 1980; Asher et al., 1981).

$InsP_6$ Effect on Heme Conformation. Addition of $InsP_6$ to $Hb^{III}F$ results in numerous X-ray, spectroscopic, and chemical differences, indicating a conversion of the protein from the R to the T quaternary form (Perutz et al., 1974a–c, 1978; Fermi & Perutz, 1977). Associated with this quaternary structural change is a 20-Å absorption spectral shift of the ca. 6000-Å charge-transfer band to longer wavelength (Figure 5). The resonance Raman spectra reported here (Figure 6) and by Rousseau et al. (1980) indicate $InsP_6$ binding induces changes in the frequencies of some heme macrocyclic modes, but the magnitude of any frequency shift for the Fe–F stretching vibration is less than 0.5 cm^{-1} and corresponds to a change of the average Fe–F bond lengths of the α and β $Hb^{III}F$ subunits of less than 0.002 Å. This suggests a similar limit for any iron movement relative to the heme plane.

The fact that the major perturbation of heme conformation is detected in the heme macrocyclic modes suggests that at least for $Hb^{III}F$ the major effect of the R $\rightarrow$ T conversion is to perturb the heme macrocycle conformation. Possibly, changes in the amino acid side chain and heme contacts occur to slightly alter heme geometry, resulting in small frequency decreases for the 760-cm^{-1} peak as well as for the 1370-cm^{-1} peak examined by Rousseau et al. (1980).

An important conclusion of this study is that although absorption spectral shifts to longer wavelengths can be correlated with an increasing out-of-plane iron distance for the isolated $Hb^{III}F$ β subunits, α subunits, and $Mb^{III}F$ a similar spectral shift of comparable magnitude occurs with $InsP_6$ *without* any detectable change in iron geometry.

Conclusions

Significant differences are found between the isolated α and β subunits of human $Hb^{III}F$ and $Mb^{III}F$ for the Fe–F stretching frequency. These differences are interpreted to result from different iron displacements to the proximal heme side in the series $Mb^{III}F$ > α subunits > β subunits. Only small differences are observed between $Hb^{III}F$ and the sum of the spectra of the isolated subunits, indicating little change in heme conformation due to the unique α–β-subunit contacts present in the tetramer. We have reexamined the effect of the R $\rightarrow$ T conformational transition induced by $InsP_6$ on $Hb^{III}F$ and find that no detectable change occurs in the iron–fluoride geometry. However, the changes observed in heme macrocyclic vibrational modes suggest that the major R $\rightarrow$ T heme conformational differences are localized in the heme macrocycle.

Acknowledgments

We thank Mary L. Adams for expert technical assistance.

References

Abe, M., Kitagawa, T., & Kyogoku, Y. (1978) *J. Chem. Phys. 69*, 4526.

Antonini, E., & Brunori, M. (1971) *Front. Biol. 21*, 1.

Asher, S. A., & Sauer, K. (1976) *J. Chem. Phys. 64*, 4115.

Asher, S. A., & Schuster, T. M. (1979) *Biochemistry 18*, 5377.

Asher, S. A., & Schuster, T. M. (1981) *Interaction Between Iron and Proteins in Oxygen and Electron Transport* (Ho, C., Ed.) Elsevier, New York (in press).

Asher, S. A., Vickery, L. E., Schuster, T. M., & Sauer, K. (1977) *Biochemistry 16*, 5849.

Asher, S. A., Adams, M., & Schuster, T. M. (1981) *Biochemistry* (in press).

Beetlestone, J., & George, P. (1964) *Biochemistry 3*, 707.

Benesch, R., Benesch, R. E., & MacDuff, G. (1964) *Biochemistry 3*, 1132.

Bucci, E., & Fronticelli, C. (1965) *J. Biol. Chem. 240*, PC551.

Bucci, E., Fronticelli, C., Chiancone, E., Wyman, J., Antonini, E., & Rossi-Fanelli, A. (1965) *J. Mol. Biol. 12*, 183.

Churg, A. K., Glick, H. A., Zelano, J. A., & Makinen, M. W. (1979) in *Biological and Clinical Aspects of Oxygen*

(Caughey, W. S., Ed.) p 125, Academic Press, New York.
Deatherage, J. F., Loe, R. S., & Moffat, K. (1976) *J. Mol. Biol. 104*, 723.
Desbois, A., Lutz, M., & Banerjee, R. (1979) *Biochemistry 18*, 1510.
Eaton, W. A., & Hochstrasser, R. M. (1968) *J. Chem. Phys. 49*, 985.
Fermi, G., & Perutz, M. F. (1977) *J. Mol. Biol. 114*, 421.
Fuchsman, W. H., & Appleby, C. A. (1979) *Biochemistry 18*, 1309.
George, P., Beetlestone, J., & Griffith, J. S. (1964) *Rev. Mod. Phys. 36*, 441.
Geraci, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4664.
Gupta, R. K., & Mildvan, A. S. (1975) *J. Biol. Chem. 250*, 246.
Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., & Gurd, F. R. N. (1968) *J. Biol. Chem. 243*, 683.
Hensley, P., Moffat, K., & Edelstein, S. J. (1975) *J. Biol. Chem. 250*, 9391.
Hoard, J. L. (1971) *Science (Washington, D.C.) 174*, 1295.
Huong, P. V., & Pommier, J.-C. (1977) *C. R. Hebd. Seances Acad. Sci., Ser. C 285*, 519.
Iizuka, T., & Kotani, M. (1969) *Biochim. Biophys. Acta 194*, 351.
Kitagawa, T., Kyogoku, Y., Iizuka, T., & Saito, M. I. (1976) *J. Am. Chem. Soc. 98*, 5169.
Koenig, S. H., Brown, R. D., & Lindstrom, T. R. (1981) *Interaction Between Iron and Proteins in Oxygen and Electron Transport* (Ho, C., Ed.) Elsevier, New York (in press).
Lanir, A., Yu, N.-T., & Felton, R. H. (1979) *Biochemistry 10*, 1656.
Makinen, M. W., Houtchens, R. A., & Caughey, W. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6042.
McCoy, S., & Caughey, W. S. (1970) *Biochemistry 9*, 2387.
McCoy, S., & Caughey, W. S. (1971) in *Probes of Structure and Function of Macromolecules and Membranes, Probes of Enzymes and Hemoproteins* (Chance, B., Yonetani, T., & Mildvan, A. S., Eds.) Vol. 2, p 289, Academic Press, New York.
Moffat, K., Deatherage, J. F., & Seybert, D. W. (1979) *Science (Washington, D.C.) 206*, 1035.
Ogawa, S., Shulman, R. G., Fujiwara, M., & Yamane, T. (1972) *J. Mol. Biol. 70*, 301.
Perutz, M. F. (1979) *Annu. Rev. Biochem. 48*, 327.
Perutz, M. F., Ladner, J. E., Simon, S. R., & Ho, C. (1974a) *Biochemistry 13*, 2163.
Perutz, M. F., Fersht, A. R., Simon, S. R., & Roberts, G. C. K. (1974b) *Biochemistry 13*, 2174.
Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., & Slade, E. F. (1974c) *Biochemistry 13*, 2187.
Perutz, M. F., Sanders, J. K. M., Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W.-M., Ho, C., Giannini, I., Porschke, D., & Winkler, H. (1978) *Biochemistry 17*, 3640.
Rousseau, D. L., Shelnutt, J. A., Henry, E. R., & Simon, S. R. (1980) *Nature (London) 285*, 49.
Smith, D. W., & Williams, R. J. P. (1970) *Struct. Bonding (Berlin) 7*, 1.
Spaulding, L. D., Chang, C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517.
Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169.
Spiro, T. G., & Burke, J. M. (1976) *J. Am. Chem. Soc. 98*, 5482.
Spiro, T. G., Stong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648.
Valdez, R., & Ackers, G. K. (1977) *J. Biol. Chem. 252*, 74.
Valdez, R., & Ackers, G. K. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) p 527, Academic Press, New York.
Warshel, A. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 273.
Williams, R. C., Jr., & Tsay, K.-Y. (1973) *Anal. Biochem. 54*, 137.
Yonetani, T., Iizuka, T., & Waterman, M. R. (1971) *J. Biol. Chem. 246*, 7683.
Yu, N.-T. (1977) *CRC Crit. Rev. Biochem. 4*, 229.