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Differences in Fe(II)-N_ϵ(His-F8) stretching frequencies between deoxyhemoglobins in the two alternative quaternary structures

(allosteric mechanism/protein dynamics/strain energy/Raman spectra/subunit heterogeneity)

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ABSTRACT **Resonance Raman spectra have been obtained of the α^{deoxy} and β^{deoxy} subunits within valency hybrid hemoglobins both in the high-affinity (R) and low-affinity (T) structures. Upon conversion from the R to the T structure, the vibrational frequency of the Fe(II)-N_ϵ(His-F8) bond changes from 223 to 207 or 203 cm^{-1} in the α^{deoxy} subunit and from 224 to 220 or 217 cm^{-1} in the β^{deoxy} subunit. We estimate that the Fe(II)-N_ϵ(His-F8) bond is stretched by the R→T transition 3 times more in the α subunit (0.02$_4$ Å) than in the β subunit (0.008$_5$ Å) and, accordingly, the strain energy developed in that bond is 8 times larger in the α than in the β subunit. Hence, the oxygen affinity of the α and β subunits may be regulated by different mechanisms.**

The oxygen affinity of Hb increases with the number of bound oxygen molecules. This increase in oxygen affinity, known as the heme–heme interaction, arises from a reversible transition between two alternative quarternary structures, the low-affinity (T) and high-affinity (R) structures (1, 2). The free energy of oxygen binding is larger by 3.6 kcal/mol (heme) in the R structure than in the T structure (3, 4). Perutz (5) proposed that the equilibrium between the two quaternary structures depends on the distance between N_ϵ(His-F8) and the plane of the porphyrin. The T structure is more stable when the iron atom is penta-coordinated and when both the iron atom and the proximal His-F8 are displaced from the porphyrin plane. The stronger bonds between the subunits in the T structure might pull the proximal His-F8 away from the porphyrin plane and thus stretch the Fe-N_ϵ(His-F8) bond. If the Fe-N_ϵ(His-F8) bond is stretched by the R→T transition, then the Fe-N_ϵ(His-F8) stretching frequency would be altered. Nagai *et al.* (6) found that the Fe(II)-N_ϵ(His-F8) stretching Raman line of deoxyHb was shifted from 221 to 216 cm^{-1} by the R→T transition. From a Morse potential function, they concluded that the Fe(II)-N_ϵ(His-F8) bond is stretched by 0.01 Å in the T structure due to the strain exerted by the globin.

In some circumstances, the α and β subunits differ spectroscopically (7–14). In deoxyHb, the α subunit is mainly responsible for the changes in the Soret and visible absorption bands observed on R→T transition (7, 8). Perutz *et al.* (15, 16) interpreted these spectral changes in terms of increased Fe(II)-N distance in the T structure. If the globin exerts a larger strain on the Fe(II)-N_ϵ(His-F8) bond in the α than in the β subunit, then the Fe(II)-N_ϵ(His-F8) stretching frequency should reveal it.

We have measured resonance Raman spectra of various valency hybrid Hbs in the T and R structures. The excitation of Raman scattering at 441.6 nm enhanced only the Raman spectra of the ferrous subunit and thus allowed us to observe the R→T-linked structural changes of the heme in the α^{deoxy} or β^{deoxy} subunit within valency hybrid Hbs. The R→T-linked frequency change of the Fe(II)-N_ϵ(His-F8) stretching Raman line was much larger in the α than in the β subunit.

MATERIALS AND METHODS

Human adult Hb (Hb A), *S*-(*N*-ethylsuccinimido)cysteinyl (NES) des-Arg$^{141\alpha}$-Hb, and the isolated α and β chains were prepared as described (17). Cyanomet hybrid Hbs were prepared as reported (18). Hb M Milwaukee and Hb M Boston were purified by ion-exchange chromatography on an Amberlite IRC-50 (type II) column equilibrated with 0.1 M phosphate buffer (pH 7.0) (19). All Hb solutions were deionized by passage through a Dintzis column (20) and deoxygenated by repeated evacuation and flushing with N_2 gas.

Raman scattering was excited by the 441.6 nm line of a He-Cd laser (Kinmon, Tokyo, Japan) and was recorded on a JEOL-400D Raman spectrometer. In order to eliminate the photoreduction of the ferric subunit by laser irradiation (21), we used a spinning cell (1800 rpm). A large amount of sample was placed in the cell to minimize the ferric heme redistribution in valency hybrid Hbs (18). The Raman spectra measured after prolonged laser irradiation (up to 5 hr) were identical to those measured immediately. This indicates negligible redistribution of the ferric heme. For some of the hybrid Hbs, the ferric heme redistribution was checked experimentally as described (18).

RESULTS

Fig. 1 shows the resonance Raman spectra of deoxyHb in the T (Hb A) and R (NES des-Arg$^{141\alpha}$-Hb) structures excited at 441.6 nm, which differ in only one respect. The former exhibited a polarized Raman line at 215 cm^{-1} and the latter at 221 cm^{-1}. No other Raman lines in the 1700–100 cm^{-1} region exhibited a frequency shift larger than 2 cm^{-1} on R→T transition. Note that the Raman line of deoxy NES des-Arg$^{141\alpha}$-Hb at 221 cm^{-1} was sharper and more symmetric than the corresponding line of deoxyHb A at 215 cm^{-1} and that the latter was resolved into two peaks by narrowing the slit-width.

Fig. 2 shows the resonance Raman spectra of the isolated α^{deoxy} chain and valency hybrid Hbs in which the α subunits are in the ferrous-deoxy and the β subunits in the ferric form. Upon excitation at 441.6 nm, the Raman spectrum of deoxyHb in this region was more strongly resonance enhanced than the spectra of any metHb derivatives. All the prominent Raman lines shown in Fig. 2 either were greatly weakened or disappeared on oxygenation of the ferrous subunit, which indicates that the ferric subunit contributed little to the spectra of the deoxygenated hybrids. Furthermore, metHb derivatives do not

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Abbreviations: Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Ins-P_6, inositol hexaphosphate; NES, *S*-(*N*-ethylsuccinimido)cysteinyl.

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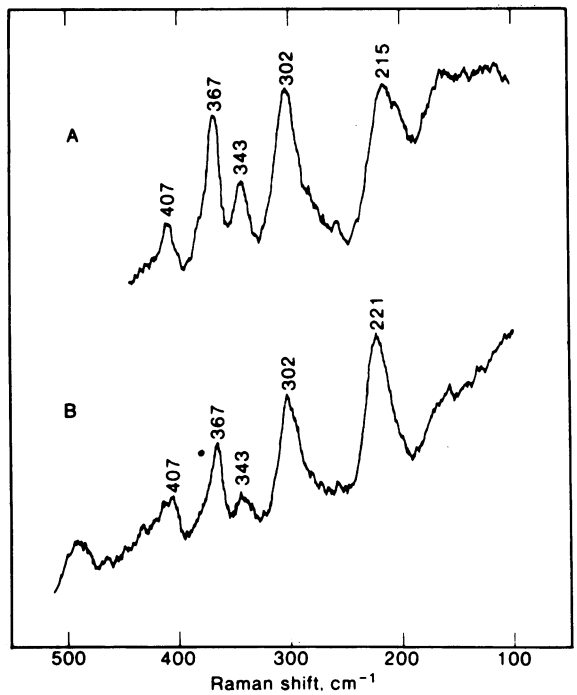

FIG. 1. Resonance Raman spectra of deoxyHb A (*A*) and deoxy NES des-Arg$^{141\alpha}$-Hb (*B*) in 50 mM [bis(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane (Bistris)/50 mM Tris buffer, pH 6.5, excited at 441.6 nm.

exhibit a prominent Raman line below 250 cm^{-1} (unpublished data). Therefore, the frequencies of Raman lines of the ferrous subunit can be accurately determined from these spectra. $\alpha_2^{deoxy}\beta_2^{+CN}$ has the R structure in the absence of inositol hexaphosphate (Ins-P_6), but can be converted to the T structure by addition of Ins-P_6 (18, 22). The Raman spectrum of $\alpha_2^{deoxy}\beta_2^{+CN}$ determined in the absence of Ins-P_6 was identical to that of the isolated α^{deoxy} chain. The Fe(II)-N$_\epsilon$(His-F8) stretching line of $\alpha_2^{deoxy}\beta_2^{+CN}$ was observed at 222 cm^{-1} in the absence of Ins-P_6, but the Raman line was shifted to 207 cm^{-1} by addition of Ins-P_6. Fig. 2 also includes the Raman spectrum of deoxyHb M Milwaukee [Val-E11(67)β→Glu] in which the iron atom in the abnormal β subunit has become ferric and the carboxyl group of Glu-E11 is coordinated to the ferric heme iron (23). The Fe(III)-O(Glu-E11) bond stabilizes the T structure and, therefore, Hb M Milwaukee with Ins-P_6 remains in the T structure even after full oxygenation (23–26). The α^{deoxy} subunit of Hb M Milwaukee exhibited the Fe(II)-N$_\epsilon$(His-F8) stretching line at 203 cm^{-1}, whereas the frequencies of other Raman lines were the same as those in the R structure. Note that the Fe(II)-N$_\epsilon$(His-F8) stretching Raman line is weaker in the T than in the R structure.

Fig. 3 shows the resonance Raman spectra of the isolated β^{deoxy} chain and valency hybrid Hbs in which the β subunit was in the deoxy form. $\alpha_2^{+CN}\beta_2^{deoxy}$ adopted the R structure in the absence of Ins-P_6, but switched to the T structure on addition of Ins-P_6 (18, 22). $\alpha_2^{+CN}\beta_2^{deoxy}$ exhibited the Fe(II)-N$_\epsilon$(His-F8) stretching line at 224 cm^{-1} in the absence of Ins-P_6 at pH 9.0 and at 220 cm^{-1} in the presence of Ins-P_6 at pH 6.5. Other Raman lines remained almost unshifted on R→T transition. Fig. 3*D* shows the Raman spectrum of deoxyHb M Boston [His-E7(58)α→Tyr], in which the iron atom in the abnormal α subunit is in the ferric form and the phenolate oxygen of Tyr-E7 is coordinated to the ferric heme iron (27). Because the molecule is frozen in the T structure by the Fe(III)-O(Tyr-E7) bond, Hb M Boston shows low oxygen affinity without heme–

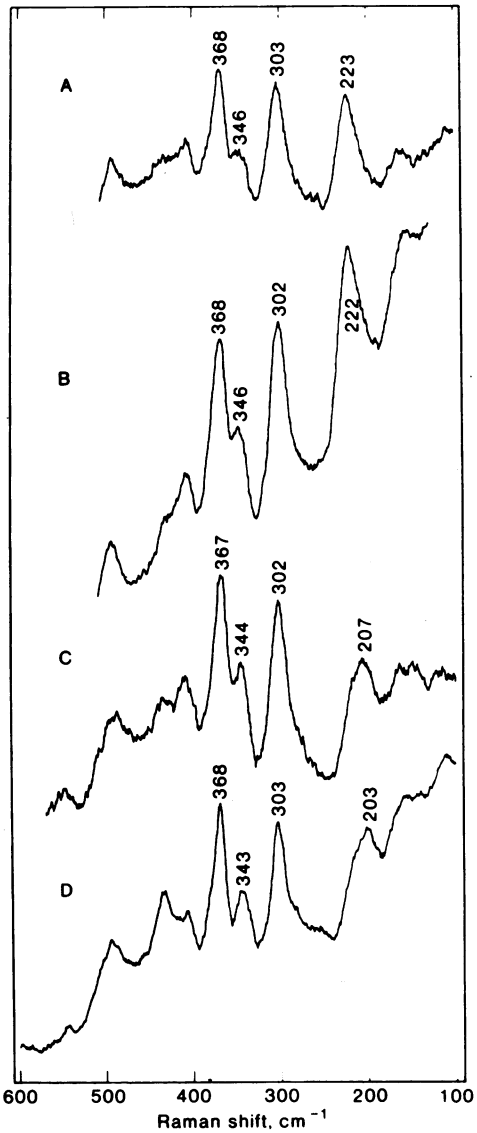

FIG. 2. Resonance Raman spectra of the isolated α chain and valency hybrid Hbs in 50 mM Bistris/50 mM Tris buffer excited at 441.6 nm. (*A*) Isolated α^{deoxy} chain at pH 6.5; (*B*) stripped $\alpha_2^{deoxy}\beta_2^{+CN}$ at pH 9.0; (*C*) $\alpha_2^{deoxy}\beta_2^{+CN}$ with Ins-P_6 at pH 6.5; (*D*) stripped deoxyHb M Milwaukee at pH 6.5.

heme interaction (27, 28). The Fe(II)-N$_\epsilon$(His-F8) stretching line of the β^{deoxy} subunit was observed at 217 cm^{-1} whereas other Raman lines appeared almost at the same frequencies as in the R structure.

In summary, the shift of the Fe(II)-N$_\epsilon$(His-F8) stretching Raman line observed on R→T transition was 3 times larger in the α^{deoxy} subunit ($\Delta\nu$ = 20 cm^{-1}) than in the β^{deoxy} subunit ($\Delta\nu$ = 7 cm^{-1}).

DISCUSSION

Assignment of Raman Line Sensitive to R→T Transition. The assignment of the R→T-sensitive Raman line is important for understanding the frequency shift. Kitagawa *et al.* (29) assigned the 220 cm^{-1} line of deoxymyoglobin to the Fe(II)-N$_\epsilon$(His-F8) stretching mode on the basis of the isotopic frequency shift upon ^{56}Fe→^{54}Fe substitution and the comparison between the penta- and hexa-coordinated heme–imidazole complexes. This assignment is now supported by several other results: the tetra-coordinated protoporphyrin Fe(II) complex in the detergent cetyltrimethylammonium bromide shows only

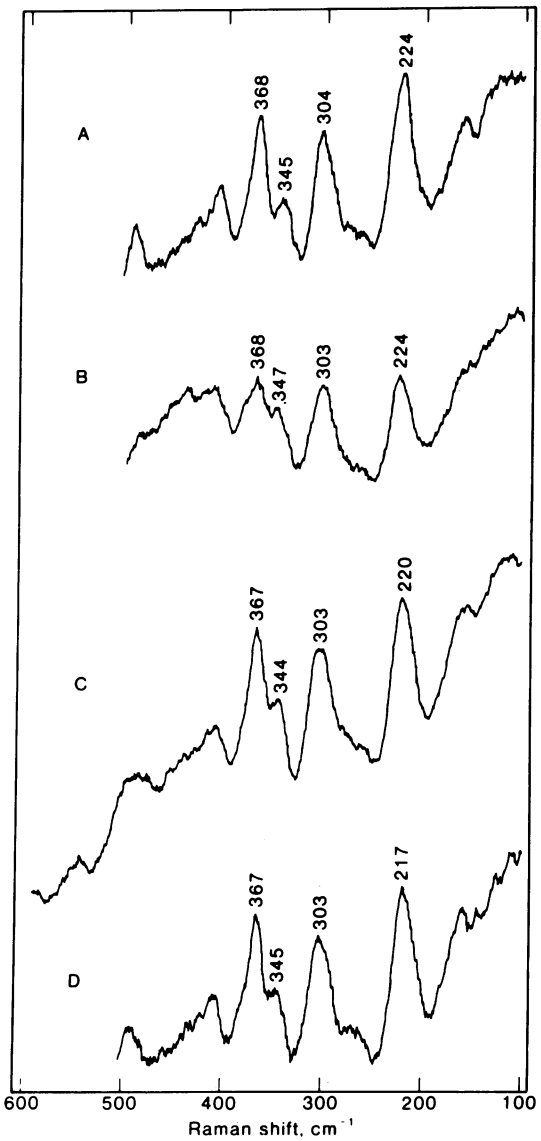

FIG. 3. Resonance Raman spectra of the isolated β chain and valency hybrid Hbs in 50 mM Bistris/50 mM Tris buffer excited at 441.6 nm. (*A*) Isolated β^{deoxy} chain at pH 6.5; (*B*) stripped $\alpha_2{}^{+CN}\beta_2{}^{deoxy}$ at pH 9.0; (*C*) $\alpha_2{}^{+CN}\beta_2{}^{deoxy}$ with Ins-P_6 at pH 6.5; (*D*) stripped deoxyHb M Boston at pH 6.5.

two lines at 417 and 384 cm^{-1}; addition of 2-methylimidazole gives rise to the line at 207 cm^{-1} (6). Nagai *et al.* (6) observed a $^{57}Fe \rightarrow {}^{54}Fe$ isotopic frequency shift of 3 cm^{-1} for the R→T-sensitive line of deoxyHb A and deoxy NES des-Arg$^{141\alpha}$-Hb compared to a calculated shift of 3.4 cm^{-1}. The ferrous iron protoporphyrin–2-methylimidazole complex, having a penta-coordinated high-spin iron as deoxyHb, exhibited the corresponding Raman line at 207 cm^{-1} in the presence of detergent. This line showed frequency shifts of 4 cm^{-1} upon $^{56}Fe \rightarrow {}^{54}Fe$ substitution (6) and of −3 cm^{-1} upon perdeuteration of 2-methylimidazole compared to a calculated shift of −2.7 cm^{-1} (30). In the absence of detergent, the corresponding line was observed at 220 cm^{-1}, probably because a water molecule binds to the imidazole. This band was also shifted upon perdeuteration of 2-methylimidazole (31). The ferrous iron picket-fence porphyrin–2-methylimidazole complex (32) exhibited the corresponding Raman line at 209 cm^{-1}, which is shifted to 212 cm^{-1} upon $^{56}Fe \rightarrow {}^{54}Fe$ substitution and to 206 cm^{-1} upon perdeuteration of 2-methylimidazole (30).

If the R→T-sensitive line were due to a totally symmetric porphyrin deformation mode as Kincaid *et al.* (31) suggest, the R→T transition would be unlikely to alter the frequency of only one porphyrin mode by as much as 20 cm^{-1}, leaving other porphyrin modes shifted by less than 3 cm^{-1}, since all the porphyrin modes involve more or less common internal coordinates. On the basis of these findings, we can be confident that the R→T-sensitive line of deoxyHb is associated primarily with the Fe(II)-N_ϵ(His-F8) stretching mode. The observed $^{57}Fe \rightarrow {}^{54}Fe$ isotopic frequency shift ($\Delta\nu$ = 3 cm^{-1}) of the Fe(II)-N_ϵ(His-F8) stretching line of deoxyHb A is close to the expected value ($\Delta\nu$ = 3.5 cm^{-1}) for the isolated Fe-imidazole stretching mode. This implies that the normal mode has more than 80% Fe(II)-N_ϵ(His-F8) stretching character.

R→T-Linked Change of Fe(II)-N_ϵ(His-F8) Bond in α^{deoxy} and β^{deoxy} Subunits. Chemical bonds of diatomic molecules are well characterized by the Morse potential defined by Eq. 1

$$V(r) = D_e\{1 - \exp[-a(r - r_e)]\}^2, \quad [1]$$

in which r_e is the equilibrium bond distance, D_e is the dissociation energy, and a is the Morse parameter. The harmonic force constant (k_e) at $r = r_e$ is given by $k_e = 2D_e a^2$.

When the Fe(II)-N_ϵ(His-F8) stretching mode is relatively isolated, it may be treated in the diatomic approximation. Because the effective mass of atoms involved in the vibration is unaltered between the T and R structures, the observed frequency shift would be ascribed to an appreciable change in the force constant. When the Fe(II)-N_ϵ(His-F8) bond is stretched by Δr due to strain exerted by the globin and the potential of globin is assumed to be linear with respect to r, the harmonic force constant at the displaced position (k_d) is represented by Eq. 2:

$$k_d = k_e(1 - 3a\Delta r). \quad [2]$$

Thus the ratio (γ) of the vibrational frequency at the displaced position (ν_d) to that at the equilibrium position (ν_e) is given by Eq. 3:

$$\gamma = (\nu_d/\nu_e) = (k_d/k_e)^{1/2} = (1 - 3a\Delta r)^{1/2}. \quad [3]$$

In our previous work (6), we assumed that the Fe(II)-N_ϵ(His-F8) bond took the equilibrium distance in the T structure because deoxyHb normally adopts the T structure. However, it appeared that the isolated α^{deoxy} (223 cm^{-1}) and β^{deoxy} (224 cm^{-1}) chains without any constraint due to bonds between subunits gave the Fe(II)-N_ϵ(His-F8) stretching Raman line almost at the same frequencies as the α^{deoxy} (222 cm^{-1}) and β^{deoxy} (224 cm^{-1}) subunits of the valency hybrid Hbs in the R structure. Therefore, it is now more reasonable to consider that $\nu_e = \nu_R$ and $\nu_d = \nu_T$. In this case, the fact that ν_R is higher than ν_T indicates positive Δr; that is, the bond is stretched in the T structure. The change in the bond length is estimated to be $\Delta r_\alpha = 0.02_4$ Å for the α^{deoxy} subunit and $\Delta r_\beta = 0.008_5$ Å for the β^{deoxy} subunit. The absolute value depends upon the Morse parameter, which is assumed to be 2.4 $Å^{-1}$ (33), but the ratio $\Delta r_\alpha/\Delta r_\beta$ [$= (1 - \gamma_\alpha{}^2)/(1 - \gamma_\beta{}^2)$] can be evaluated with certainty from the observed quantities: Δr_α is about 3 times larger than Δr_β.

The strain energy developed in the Fe(II)-N_ϵ(His-F8) bond of the α^{deoxy} (ΔV_α) and β^{deoxy} (ΔV_β) subunits can be evaluated by substituting Δr_α or Δr_β into Eq. 1. With a plausible value of D_e (= 10 kcal/mol) (34), we obtain ΔV_α = 31 and ΔV_β = 4 cal/mol (heme). The value again depends on both the Morse parameter and dissociation energy (D_e), but their ratio depends only on the experimentally determined values. For small Δr, it is represented by Eq. 4:

$$(\Delta V_\alpha/\Delta V_\beta) = (\Delta r_\alpha/\Delta r_\beta)^2. \quad [4]$$

Consequently ΔV_α is 8 times larger than ΔV_β.

Implications for Molecular Mechanism of Heme–Heme Interaction. Recently, Shelnutt *et al.* (35) observed appreciable frequency differences of the Raman lines of deoxyHb in the 1300–1600 cm^{-1} region between the T and R structures. They concluded that the R structure has an effective increase in the electron density of the antibonding π^* orbitals of the porphyrin ring. When the electron donor is Fe^{2+}, the charge transfer would be easier with shorter Fe-N(pyrrole) bond and, thus, in the R structure with the relaxed Fe-N_ϵ(His-F8) bond. Conversion of deoxyHb from the R to the T structure is accompanied by changes in absorption spectra in the visible and Soret region (15, 36, 37). Perutz *et al.* (15, 16) interpreted them in terms of increased Fe(II)-N_ϵ(His-F8) distance in the T structure. Experiments with cyanomet and proto-meso hybrid Hbs (7, 8) demonstrated that the α subunit is mainly responsible for the spectral change observed on R→T transition, which suggests that stretch of the Fe(II)-N_ϵ(His-F8) bond occurs mainly in the α subunit. We have proved that stretch of the Fe(II)-N_ϵ(His-F8) bond is in fact larger in the α^{deoxy} subunit ($\Delta r = 0.02_4$ Å) than in the β^{deoxy} subunit ($\Delta r = 0.008_5$ Å).

Because globin imposes a larger strain on the Fe(II)-N_ϵ(His-F8) bond in the α subunit, one might suspect that the differences in oxygen affinity between the T and R structures may be larger in the α than in the β subunit. However, the oxygen affinities of the α and β subunits are lowered equally on going from the R to the T structure: the differences in oxygen affinity between the isolated α chain and Hb M Milwaukee corresponds to 3.4 kcal/mol(heme) in free energy and that between the isolated β chain and Hb M Boston to 3.5 kcal/mol(heme) (refs. 17, 25, and 28; unpublished data). This suggests that the oxygen affinity of the β subunit is regulated by another mechanism in addition to the force imposed on the Fe(II)-N_ϵ(His-F8) bond. Perutz (38) suggested that restraint of the heme iron's movement may be dominant in regulating the oxygen affinity of the α subunit and steric hindrance of the ligand site by distal Val-E11 in the β subunit. Even in the α subunit, the amount of strain energy developed in the Fe(II)-N_ϵ(His-F8) bond [31 cal/mol(heme)] is much less than the free energy of cooperation [3.6 kcal/mol(heme)]. There must be a path that links the movement of the iron atom to the surface of the subunit ($\alpha_1\beta_2$ contact) where the major change in quaternary structure occurs. Presumably the rest of the free energy of cooperation is stored in the path as strain energy in hydrogen bonds, van der Waals contact, and small torsions. If each distortion took up 30 cal/mol, which in ethane corresponds to distortion energy of the C-C internal rotation by 2 degrees, and each amino acid underwent two such distortions, 60 amino acids would be involved. The number seems reasonable considering the three-dimensional structure of the Hb molecule.

When the iron atom moves into the plane of the porphyrin, the R structure becomes thermodynamically more stable than the T structure (5). In the T liganded structure, globin pulls the proximal His-F8 away from the porphyrin plane and the Fe(II)-N_ϵ(His-F8) bond is stretched from its equilibrium bond length. The strain energy thus developed would make the T liganded form unstable. The measurements of thermal spin equilibria in azide metHb by Perutz and coworkers (39, 40) showed that in Hb M Milwaukee the strain energy amounts to >0.8 kcal/mol(heme) and in carp to 1 kcal/mol(heme) averaged over the tetramer. However, it may well be unevenly distributed so that the major part, nearer 2 kcal/mol(heme), is concentrated to the α hemes. The effect of the globin structure on the Fe(II)-N_ϵ(His-F8) bond is most pronounced in nitrosylHb, where conversion to the T structure tears the Fe(II)-N_ϵ(His-F8) bond in the α subunit but not in the β subunit (9–12). This implies that the globin exerts a larger strain on the Fe(II)-N_ϵ(His-F8) bond in the α than in the β subunit, in accord with the present result. Thus, the free energy of cooperation may be stored in part in the T liganded form. Baldwin and Chothia (41) pointed out that steric strain between $C_\epsilon H$ of His-F8 and pyrrole nitrogen in the T liganded form may be responsible for part of the energy.

Another interesting feature is the dependence of the Fe-N_ϵ stretching frequency of the ferrous hemes, not only on the quaternary structure of the tetramer, but also on the tertiary structure of the ferric subunits. In the hybrid $\alpha_2^{deoxy}\beta_2^{+CN}$, in the presence of Ins-P_6, the β chains have the tertiary oxy (r) structure and the Fe_α^{2+}-N_ϵ stretching frequency is 207 cm^{-1}. In Hb M Milwaukee, the β chains have the tertiary deoxy (t) structure and the Fe_α^{2+}-N_ϵ stretching frequency is 203 cm^{-1}. Similarly, in the hybrid $\alpha_2^{+CN}\beta_2^{deoxy}$, in the presence of Ins-P_6, the α chains have the tertiary r structure and the Fe_β^{2+}-N_ϵ stretching frequency is 220 cm^{-1}, whereas in Hb M Boston the α chains have the tertiary t structure and the Fe_β^{2+}-N_ϵ stretching frequency is 217 cm^{-1}. This shows that heme–heme interaction does take place within the quaternary T structure, so that transition from the tertiary t to the tertiary r structure of the ferric subunits relaxes some of the tension in the Fe-N_ϵ bonds in the ferrous subunits. There is no evidence for any such effect in the quaternary R structure.

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