Correlation between the Iron–Histidine Stretching Frequencies and Oxygen Affinity of Hemoglobins. A Continuous Strain Model

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Abstract: The iron–histidine (F8) stretching Raman lines of various mutant deoxyhemoglobins (deoxyHb) with varied levels of oxygen affinity were observed. The oxygen binding equilibrium curves were obtained from the same preparations as those used for the Raman measurements, and three MWC parameters as well as K_1 were determined. The high-affinity deoxyHb gave the Fe–histidine stretching Raman line at 220–222 cm^{-1} similar to that of the isolated chains and deoxyMb, while the low-affinity one gave the corresponding Raman line at 214–216 cm^{-1}, in agreement with the previous observations. However, deoxyHb's with intermediate levels of oxygen affinity unexpectedly exhibited the Fe–histidine stretching mode at intermediate frequencies as a single peak. This could not be interpreted as an equilibrium mixture of the high- and low-affinity species as postulated by the simple two-state model. The variation of the Fe–histidine stretching frequencies was interpreted, in the present study, in terms of the strain imposed on the Fe–histidine bond by the globin and was correlated with the dissociation constant of oxygen which leaves oxyhemoglobin last (K_1). The plots of the observed Fe–hisitidine frequencies vs. log K_1 were found to fit surprisingly well the expected function of log K_1.

Cooperative oxygen binding of hemoglobin (Hb) has been understood in terms of the reversible transition between the two alternative quaternary structures, namely, the T state with low affinity and the R state with high affinity.[1,2] The practical structural mechanism by which the R–T transition affects the O_2 affinity of the heme iron is currently under spectroscopic studies.[3,4] Perutz[5] proposed that stronger intersubunit interactions in the T state might pull the proximal histidine (F8) away from the porphyrin plane through a tensed structure of the polypeptide skeleton and thus stretch the Fe–His(F8) bond. This idea postulates that, although the Fe–His bond is destabilized by strain from the protein, the stabilization of a whole Hb molecule attained by the stronger intersubunit hydrogen bonds and salt bridges overcomes the destabilization of the Fe–His bond in the deoxyHb. The presence of such strain in the Fe–His bond of the deoxyHb with low affinity was previously demonstrated by the observation of the R–T difference of the Fe–His stretching frequency,[6] which was confirmed by other groups later.[7,8] The R–T frequency difference was once ascribed to the difference in the strength of hydrogen bonding of the proximal histidine,[9] but inadequacy of this interpretation was pointed out later.[7] Furthermore, the analysis of the NMR signal of the proximal histidine also supported the stretch of the Fe–His bond in the low–affinity deoxyHb.[10]

The oxygen-binding equilibrium measured for a single Hb preparation under a certain solution condition can usually be well reproduced by substituting appropriate values for the three MWC parameters.[11] The free energy of cooperativity is determined to be 3.4 kcal/mol (heme) for the stripped Hb A at pH 7.4 in the presence of Cl^- ion.[12,13] In this regard the simple two-state model seems quite satisfactory and of practical importance. However, there is another suggestion that two kinds of tertiary structures, that is, deoxy and ligated conformations, should be considered for each of the quaternary states.[14] We are curious to know whether such a limited number of states as implicated by the two quaternary states or their combinations with two tertiary structures are the sole allowed structures which are common to all tetrameric hemoglobins or are the allowed states continuously distributed depending on the primary structure of the proteins. To answer this question we undertook the present study.

The Fe–His stretching vibration (ν_{Fe-His}) of hemoproteins was noticed as a sensitive structural probe of the heme vicinity.[15a–c]

The ν_{Fe-His} Raman lines of deoxyMb[16,17] and reduced peroxidases[18a,b] are observed at 222 and 244 cm^{-1}, respectively, and their frequency difference was ascribed to the strong hydrogen bond of the proximal histidine of the latter to a neighboring amino acid residue.[18a] The ν_{Fe-His} mode of the aa_3-type cytochrome oxidases, which was assigned recently to the Raman line around 213–220 cm^{-1} on the basis of the observed ^{54}Fe isotopic frequency shift,[19] was found to be extremely sensitive to a protein structure and possibly indicative of the proton pump activity of this enzyme.[20] Accordingly, it is likely that the delicate changes of the intersubunit interactions of Hb is transmitted to the heme moiety

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through the Fe–His bond, and the ν_{Fe-His} Raman line can probe them.

The ν_{Fe-His} Raman line of deoxyHb appears at 220–222 cm⁻¹ for the typical R state and at 214–216 cm⁻¹ for the typical T state,[6,7] although deoxy des-His(146β)-Arg(141α) Hb in the presence of 2 mM inositol hexaphosphate (IHP) at pH 6.5 and deoxy des-Arg(141α) Hb in the absence of IHP at pH 6.5 gave the Raman line at the intermediate frequency (218 cm⁻¹).[6] If there were no other states besides the universal T and R states, deoxyHb's with moderate levels of oxygen affinity should exhibit either two distinct Raman lines simultaneously at 220–224 and 214–216 cm⁻¹ or their unresolved composite with an apparent single peak at a frequency in between. In either case, the relative contribution of the two Raman lines should be given by the relative populations of the two species determined by the MWC parameter ($=L$). Alternatively, if a size of the strain imposed to the Fe–His bond were specific to individual molecules, each deoxyHb with different levels of oxygen affinity should give a single ν_{Fe-His} Raman line at varied frequencies depending on the magnitude of the strain.

Friedman et al.[21a,b] investigated the transient deoxyHb's occurring within 10 ns of photolysis of COHb. The T-state transients have frequencies ranging from 216 to 222 cm⁻¹ whereas the R-state transients range from 222 to 232 cm⁻¹, which are higher than those of the corresponding deoxy species in equilibrium. The transient state with the ν_{Fe-His} mode at higher frequencies remains as a stable species at cryogenic temperatures.[22a,b] The ν_{Fe-His} frequencies of transient deoxyHb's of various sources are distributed almost continuously[23] and appear to correlate with parameters of ligand-binding reactivity including geminate recombination and off rates.[21a] These facts may suggest the presence of other states with varied magnitude of strain in the Fe–His bond besides the two typical states.

The oxygen affinity of a variety of mutant Hb's having various levels of oxygen affinity was systematically investigated, and a linear correlation was found between log c and log L,[24] where c ($=K_R/K_T$) and L are the MWC parameters. Imai[25] has also found the parameter correlation and demonstrated that most of the oxygen-binding curves of a single Hb in various conditions could be well simulated by manipulating only one parameter, namely K_T which is practically the same as K_1. If the oxygen affinity is significantly regulated by the strain imposed on the Fe–His(F8) bond of the deoxy form, the magnitude of the strain is expected to have some correlation with oxygen affinity. Accordingly, we investigated various mutant Hb's and measured the resonance Raman (RR) spectra and oxygen binding equilibria for the same preparation, aiming at correlation of their ν_{Fe-His} frequencies with the K_1 constant.

Experimental Section

Measurements of the RR Spectra. Raman spectra were obtained with the 441.6-nm line of a He/Cd laser (Kinmon Electrics, CDR80SG) and a JEOL-400D Raman spectrometer equipped with a cooled RCA-31034a photomultiplier. Approximately 0.3 mL of 400 μM (heme) Hb solution was placed in a cylindrical cell sealed with a rubber septum for evacuation and was kept at 10 °C during the measurements. The frequency calibration of the spectrometer was performed with CCl_4 as a standard. Absence of the oxygenated or the met form was confirmed every time by observing the single ν_4 line around 1355 cm⁻¹. The pH value of the Hb solution was determined with a Beckman-ϕ71 pH meter at 10 °C immediately after the Raman measurements.

Purification of Hemoglobin and Chains. Hb A was purified from normal blood with CM-32 column chromatography. The preparation procedures of Hb Kempsey (β99Asp→Asn),[26] Hb J Capetown

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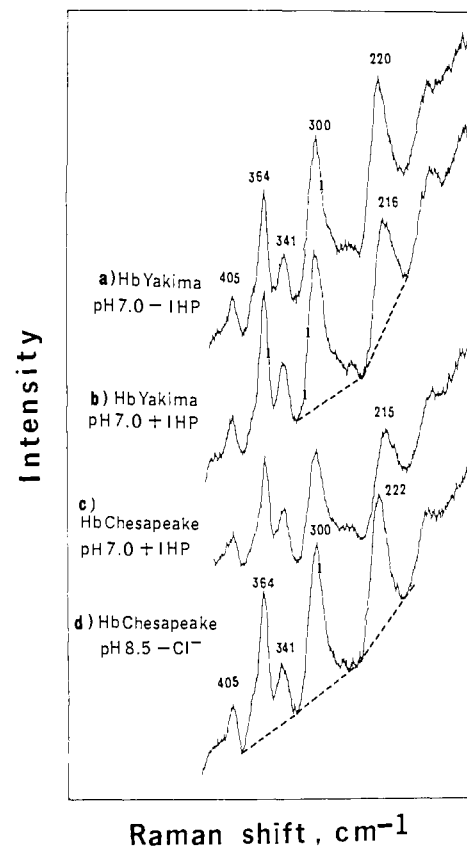

Raman shift, cm⁻¹

Figure 1. Typical RR spectra of deoxyHb's with high affinity (a and d) and low affinity (b and c): (a) Hb Yakima in the absence of IHP, (b) Hb Yakima in the presence of 1 mM IHP, (c) Hb Chesapeake in the presence of 1 mM IHP, (d) Hb Chesapeake in the absence of IHP. The solvents for a, b, and c are 0.05 M bis–tris/acetate buffer, pH 7.0, containing 0.1 M NaCl, but that for d is 0.05 M tris/acetate buffer, pH 8.5 without NaCl. The broken lines stand for the assumed base lines. Instrumental conditions: laser, 441.6 nm, 40 mW; sensitivity, 500 pulses/s; slit width, 4.5 cm⁻¹; time constant, 8 s; scan speed, 10 cm⁻¹/min.

(α92Arg→Glu),[26] Hb Hirose (β37Trp→Ser),[27a] Hb Kansas (β102Asn→Thr),[27b] and isolated chains of Hb A[27a] were reported previously. Hb Yakima (β99Asp→His) and Hb Chesapeake (α92Arg→Leu) were purified from the patient's blood with DE-32 column chromatography.[26] The purified mutant Hb's were stored in the CO bound form at 4 °C until the Raman spectra and oxygen-binding equilibria were measured. The isoelectric focusing on a LKB plate (pH 3.5 to 9.5) confirmed that these Hb's are essentially free from the half-oxidized forms.

The Hb solutions were passed through a Sephadex G-25 column equilibrated with the 0.01 M bis–tris/acetate buffer, pH 7.0 to change a buffer, and the eluate was concentrated to ~1.2 mM (heme) with an Amicon ultrafiltration apparatus with a PM-10 membrane. Organic phosphate bound to Hb was completely removed through these column treatments. The stock solution was diluted to 400 μM by addition of appropriate amounts of the NaCl (1 M), IHP (50 mM), and bis–tris/acetate or tris/acetate buffer. Heme concentration was determined with the method of pyridine hemochrome with $\epsilon_{mM} = 34$ cm⁻¹.

Determination of K_1. Oxygen equilibrium curves of mutant Hb's under several solvent conditions were determined with the automatic recording apparatus described previously.[24] The K_1 values were given as the intercept on the pO_2 axis intersected by the lower asymptote of Hill plots for the oxygen-binding equilibrium and represented as the dissociation constant of oxygen which leaves oxyHb last.

Results

Figure 1 shows typical RR spectra of deoxyHb's with low or high affinity. Hb Yakima in the absence of IHP at pH 7 gave

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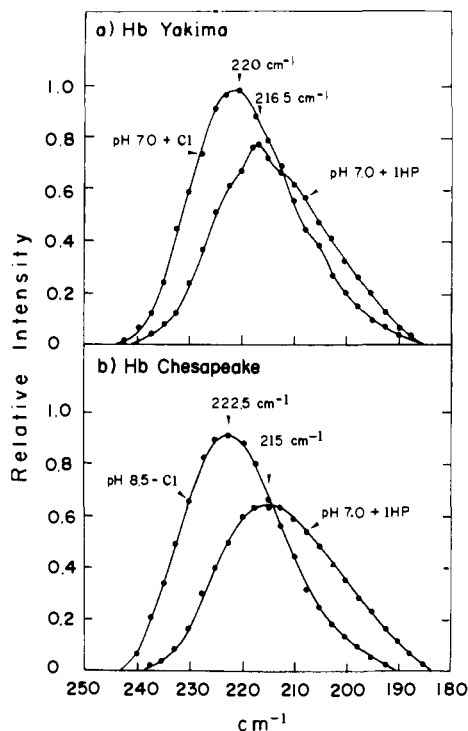
Figure 2. Base line subtracted band shape of the Fe–His stretching Raman lines of deoxyHb Yakima (a) and deoxyHb Chesapeake (b). The assumed base lines are represented by broken lines in Figure 1. The peak heights of all the Raman lines are normalized by the base line subtracted Raman line at 300 cm^{-1}. The ordinate scale stands for the relative value to the intensity of the Raman line at 300 cm^{-1} of the individual spectra.
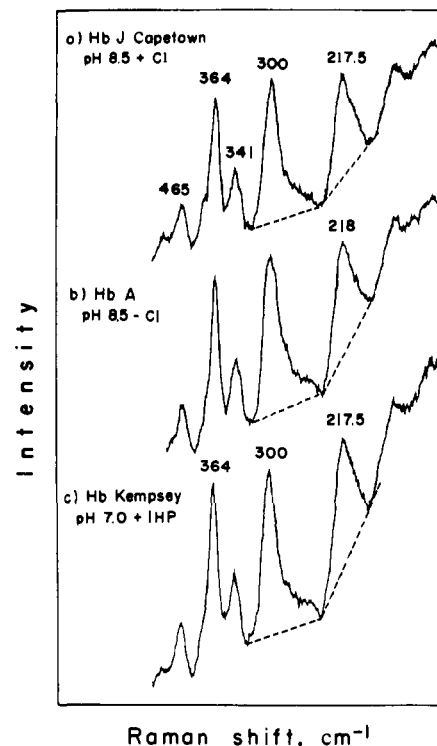

Figure 3. The RR spectra of deoxyHb's with intermediate levels of oxygen affinity: (a) Hb J Capetown in the presence of 0.1 M NaCl at pH 8.5, (b) Hb A in the absence of chloride at pH 8.5, (c) Hb Kempsey in the presence of 1 mM IHP at pH 7.0. The broken lines indicate the assumed base lines. The instrumental conditions are the same as those in Figure 1.

K_1 = 1.02 mmHg and $\nu_{\text{Fe-His}}$ = 220 cm^{-1} (a), but the same protein in the presence of IHP gave K_1 = 26.9 mmHg and $\nu_{\text{Fe-His}}$ = 216 cm^{-1} (b). Similarly, Hb Chesapeake in the high-affinity state gave K_1 = 0.60 mmHg and $\nu_{\text{Fe-His}}$ = 222 cm^{-1} (d), but that in the low-affinity state gave K_1 = 37.1 mmHg and $\nu_{\text{Fe-His}}$ = 215 cm^{-1} (c), while the frequencies of other modes were little altered. Note that the separated α chain gave K_1 = 0.6 mmHg and $\nu_{\text{Fe-His}}$ = 223 cm^{-1} similar to the high-affinity Hb Chesapeake.

One may argue that the overlapped tail of the Rayleigh scattering may cause a shift of the lower frequency peak. Figure 2 plots the base line subtracted band shape of the $\nu_{\text{Fe-His}}$ modes, which was obtained by normalizing the base line subtracted intensity with regard to that at 300 cm^{-1}. The peak frequencies were altered at most by 0.5 cm^{-1} by these base line corrections. It is noted that the high affinity form yields a more symmetric band shape with appreciably higher peak height even after normalization.

Figure 3 shows the observed RR spectra of deoxyHb's having intermediate levels of oxygen affinity. The base line subtracted band shape of the $\nu_{\text{Fe-His}}$ RR lines is depicted in Figure 4. Hb J Capetown in the presence of 0.1 M Cl$^-$ at pH 8.5 gave K_1 = 5.0 mmHg and $\nu_{\text{Fe-His}}$ = 217.5 cm^{-1} (a). Hb A in the absence of Cl$^-$ at pH 8.5 gave K_1 = 4.9 mmHg and $\nu_{\text{Fe-His}}$ = 218 cm^{-1} (b). Hb Kempsey in the presence of 1 mM IHP at pH 7.0 gave K_1 = 14.65 mmHg and $\nu_{\text{Fe-His}}$ = 217.5 cm^{-1} (c). It is stressed that these spectra cannot be a weighted sum of the two spectra of the typical T and R forms shown in Figure 2.

The spectral patterns of other Hb's were more or less similar to those in Figures 1 or 3. Their $\nu_{\text{Fe-His}}$ frequencies were determined from the base line subtracted spectra and listed in Table I together with K_1. The MWC parameters for these Hb's obtained previously[24] are also included in Table I. The $\nu_{\text{Fe-His}}$ frequencies become higher as the oxygen affinity becomes higher. This tendency is more clearly recognized in the graphic display shown in Figure 5, where the $\nu_{\text{Fe-His}}$ frequencies are plotted against the K_1 constant. The similar trend between the Raman frequencies and P$_{50}$ of some monomeric or modified Hb's were previously noted,[8] although the assignment of the Raman line was different.
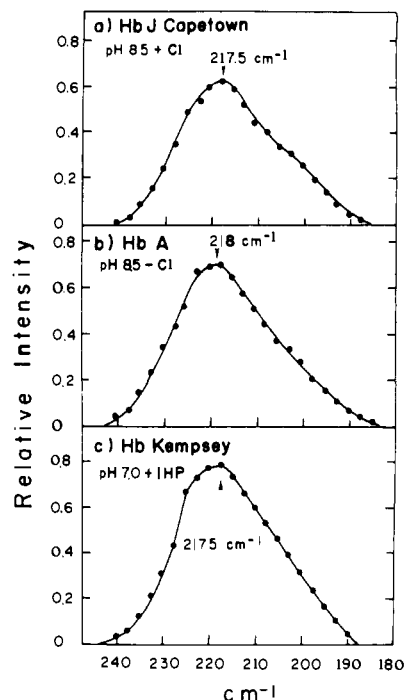
Figure 4. The base line subtracted band shape of the Fe–His stretching Raman line of deoxyHb J Capetown (a), deoxyHb A (b), and deoxyHb Kempsey (c). The assumed base lines are represented by broken lines in Figure 3. The ordinate scale stands for the relative intensity to the Raman line at 300 cm^{-1} of the individual spectra.

Discussion

The simple two-state model postulates the presence of only two states, namely, the typical T and R states. One who strictly adheres to this theory might draw a line at 218 cm^{-1}, classifying those above and below this line as the R and T states, respectively.

Table I. Observed Values of $\nu_{\text{Fe–His}}$ and K_1 of Several Hemoglobins

no.	name/conditions	$\nu_{\text{Fe–His}}{}^a$	$K_1{}^b$	L^f	c	K_T	K_R
1	Hb A/pH 7.0, +IHP^c	214.5	138	$4.5 \times E8$	0.0029	138	0.40
2	Hb A/pH 7.0, +Cl^d	215	70	$4.35 \times E7$	0.002	70	0.14
3	Hb A/pH 8.5, +Cl	216	12.5	$5.0 \times E3$	0.008	12.5	0.10
4	Hb A/pH 7.0, –Cl^e	216	6.06	$9.0 \times E3$	0.033	6.06	0.20
5	Hb A/pH 8.5, –Cl	218	4.9	$9.5 \times E2$	0.050	5.0	0.25
6	Hb J Capetown/pH 7.0, +IHP	214.5	58	$9.5 \times E6$	0.0096	58	0.40
7	Hb J Capetown/pH 7.0, +Cl	215	16.7	$2.0 \times E5$	0.012	16.7	0.20
8	Hb J Capetown/pH 8.5, +Cl	217.5	5.0	$5.5 \times E3$	0.04	5.0	0.20
9	Hb J Capetown/pH 8.5, –Cl	220	1.0		n.d.		
10	Hb Chesapeake/pH 7.0, +IHP	215	37.1	$2.5 \times E5$	0.0166	37.1	0.60
11	Hb Chesapeake/pH 7.0, +Cl	216	3.52	$2.5 \times E2$	0.11	3.64	0.40
12	Hb Chesapeake/pH 8.5, +Cl	220	1.05	$1.2 \times E1$	0.33	1.21	0.40
13	Hb Chesapeake/pH 8.5, –Cl	222.5	0.60		n.d.		
14	Hb Kansas/pH 7.0, +Cl	214	50		n.d.		
15	Hb Kansas/pH 8.5, –Cl	214	19		n.d.		
16	Hb Yakima/pH 7.0, +IHP	216.5	26.9	$5.0 \times E3$	0.026	26.9	0.70
17	Hb Yakima/pH 7.0, +Cl	220	1.02	$2.0 \times E1$	0.36	1.11	0.40
18	Hb Kempsey/pH 7.0, +IHP	217.5	14.65	$9.0 \times E1$	0.070	17.1	1.20
19	Hb Kempsey/pH 7.0, +Cl	220	1.06	$2.0 \times E1$	0.30	1.33	0.40
20	Hb Hirose/pH 7.0, +IHP	218	11.5		n.d.		
21	Hb Hirose/pH 7.0, +Cl	219	2.0		n.d.		
22	Hb Hirose/pH 8.5, +Cl	219	1.3		n.d.		
23	α chain	223	0.6				
24	β chain	224	0.34				

^{a}cm^{-1}. ^{b}[mmHg]. ^{c}In the presence of 1 mM IHP. ^{d}In the presence of 0.1 M NaCl. ^{e}In the absence of chloride ion. ^{f}En means 10^n.
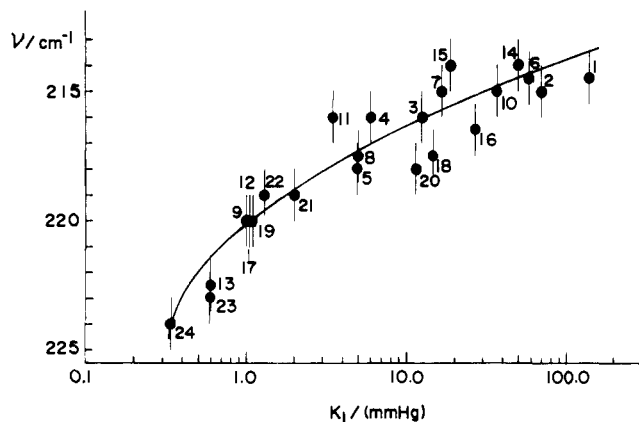

Figure 5. The $\nu_{\text{Fe–His}}$ vs. K_1 plot of various deoxyHb's. The solid line denotes the curve calculated with $\nu = 225.2 - 7.10 \, (\log K_1 - 0.488)^{1/2}$ (see text). K_1 values were determined as the intercept on the pO$_2$ axis intersected by the lower asymptote of the Hill plot for oxygen equilibrium curves reported separately[24] and represented as a dissociation constant for oxygen which leaves oxyHb last. The experimental conditions for the Raman spectra are the same as those in Figure 1. The numbers stand for the samples specified in Table I.

In this case one has to admit that both the T and R states exhibit appreciable variability. Alternatively, one may argue that the RR line appearing around 217–219 cm^{-1} is a composite of two bands which should have the band centers at 214–216 and 220–221 cm^{-1}. In this case the ratio of the contribution of the two bands should be consistent with the value of L determined from the oxygen equilibrium curve. When L is larger than 20, however, the contribution from the minor component to the apparent spectrum would be practically negligible. Accordingly, the band shape of the $\nu_{\text{Fe–His}}$ Raman line, which appears at the intermediate frequency region, cannot be explained with reasonable values of L (Table I) determined from the oxygen-binding equilibria.

The observed isotopic frequency shift of the $\nu_{\text{Fe–His}}$ mode,[6,28] suggested that this vibration is fairly isolated from the porphyrin vibrations. Moreover, for the mutant deoxyHb's examined, a frequency shift correlated with the oxygen affinity was not recognized for other low-frequency modes. Therefore, it is unlikely that the frequency shift of the $\nu_{\text{Fe–His}}$ mode results from a change of vibrational coupling with other porphyrin modes. The $\nu_{\text{Fe–His}}$
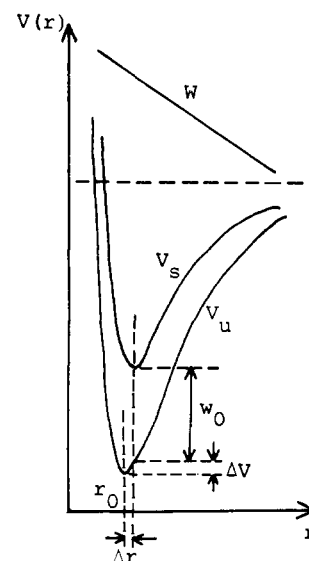
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Figure 6. Schematic diagram for the potential function of the Fe–His-(F8) bond: r, Fe–His bond length; $V_u(r)$, an intrinsic potential function of the Fe–His bond without any external strain; r_0, the equilibrium bond length of the unperturbed Fe–His bond; $W(r)$, and additional potential imposed on the Fe–His bond by the globin moiety; Δr, a stretch of the Fe–His bond in the strained structure; ΔV, increase of the energy of the Fe–His bond due to the strain.

frequency of deoxyMb is almost the same as those of the isolated chains of Hb A and also as the Fe^{2+}–1MeIm stretching frequency of the 1-methylimidazole (1MeIm) complex of ferrous iron porphyrin in an organic solution.[28] It therefore seems reasonable to assume that the $\nu_{\text{Fe–His}}$ frequency is primarily determined by an intrinsic potential of this bond for the monomeric proteins but is lowered than it by some perturbation from the protein moiety for the low-affinity tetrameric proteins. When the perturbation results in a strain of the Fe-histidine bond, it would cause a change of the force constant of the Fe–His stretching vibration as explained below.

The potential function of the Fe–His bond in the unstrained structure, $V_u(r)$, is regarded as the same as its intrinsic potential, $U(r)$, with a minimum at $r = r_0$. When the intersubunit interaction becomes stronger, the Fe–His bond will have an additional contribution of potential from the globin moiety, $W(r)$, and as a result, the bond will be strained.

$$V_u(r) = U(r) \tag{1}$$

$$V_s(r) = U(r) + W(r) \tag{2}$$

The potential of the strained Fe–His bond is represented by $V_s(r)$, which would have the minimum at $r = r_0 + \Delta r$ due to strain as illustrated in Figure 6.   The harmonic force constants for the Fe–His stretching vibration in the unstrained (k_u) and the strained conditions (k_s) are represented as

$$k_u = (d^2V_u/dr^2)_{r_0} = (d^2U/dr^2)_{r_0} \tag{3}$$

$$k_s = (d^2V_s/dr^2)_{r_0+\Delta r} \tag{4}$$

Here $(dU/dr) = -(dW/dr)$ at $r = r_0 + \Delta r$. We develop $W(r)$ at r_0 in a power series of Δr and, in the first approximation, we adopt until the linear term, that is

$$W(\Delta r) = w_0 + w_1\Delta r \tag{5}$$

and k_s is also developed at r_0 in a power series of Δr as $k_s(r_0 + \Delta r) = k_s(r_0) + (dk_s/dr)_{r_0}\Delta r$, then eq 4 can be rewritten as

$$k_s = -k_u + V'''\Delta r \tag{6}$$

where V''' is the third derivative of V_s at r_0.

The energy of the Fe–His bond in the unstrained and strained states are $V_u(r_0)$ and $V_s(r_0 + \Delta r)$, respectively.   Since the linear term in the latter is cancelled between U and W, $V_s(r_0 + \Delta r)$ is equal to $V_s(r_0) + (k_s/2)(\Delta r)^2$ in the harmonic approximation. We define ΔV as the energy difference between the Fe–His bonds of the unstrained and strained deoxyHb's.

$$\Delta V = V_s(r_0 + \Delta r) - V_u(r_0) - w_0 \tag{7a}$$

$$\Delta V = (k_s/2)(\Delta r)^2 \tag{7b}$$

Then ΔV is related with the magnitude of the bond stretch through the equation

$$\Delta r = (2\Delta V/k_s)^{1/2} \tag{8}$$

Combining eq 6 and 8 provides the equation

$$k_u - k_s = -V'''(2\Delta V/k_s)^{1/2} \tag{9}$$

Since the reduced mass, μ, of the oscillator is common to the unstrained and strained states, eq 9 can be rewritten as

$$\mu(\nu_u^2 - \nu_s^2) \simeq 2\mu\nu_s\Delta\nu = -V'''(2\Delta V/k_s)^{1/2} \tag{10}$$

where $\Delta\nu = \nu_u - \nu_s$. The value of $\Delta\nu$ is positive since the third derivative of the potential function is usually negative. Thus the "u"–"s" frequency shift is proportional to the square root of the energy increase as represented by

$$\Delta\nu = (V'''^2/2\mu k_s^2)^{1/2}(\Delta V)^{1/2} \tag{11}$$

Previously Friedman et al.[23] suggested a possibility that tilting of the Fe–His bond from the heme normal lowers the Fe–His stretching frequency as well as the oxygen affinity. On the other hand, Champion and co-workers[29] proposed that the rotation of the tilted Fe–His bond around the heme normal alters the electron population in the antibonding orbital of the Fe–His bond (σ^*_{Fe-His}) and changes the Fe–His stretching frequency. They interpreted the lower intensity of the ν_{Fe-His} RR line in the lower affinity state in terms of less coupling between σ^*_{Fe-His} and the LUMO of porphyrin.

Generally the vibrational frequencies depend upon the potential function and a molecular geometry. A change of the latter influences the frequencies through a change of effective masses. However, since the ν_{Fe-His} mode is fairly isolated as explained before, it seems unlikely that the effective mass of the Fe–His stretching mode directly depends upon the tilting angle or the rotational angle of the Fe–His bond. If the tilting or rotation of the Fe–His bond changes the Fe–His stretching force constant,

(29) Bangharoenpaurpong, O.; Schomacker, K. T.; Champion, P. M. *J. Am. Chem. Soc.* **1984**, *106*, 5688–5698.
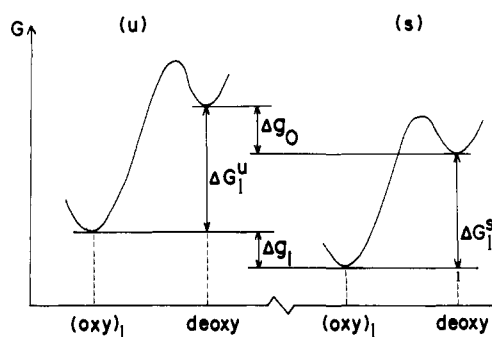

Figure 7. Schematic free energy diagram for a change from deoxy to monooxy states. (s) and (u) represent the strained and unstrained structures, respectively. ΔG_1^s and ΔG_1^u are the free energy change upon oxygenation within the strained and unstrained structures, respectively. Δg_0 and Δg_1 are the free energy differences between the strained and unstrained structures in the deoxy and monooxy states, respectively.

it implies a change of the potential function of the Fe–His bond itself, and consequently the essential point of discussion would be the same as the treatment described above except for the fact that the origin of $W(r)$ is not specified in this treatment.

On the other hand, the equilibrium constant, K_1, is related with the free energy difference between the mono-oxy and deoxy states through the equation

$$RT \ln K_1 = \Delta G_1 \tag{12}$$

As illustrated in Figure 7, the free energy changes within the unstrained and strained structures are represented by ΔG_1^u and ΔG_1^s, respectively. Suppose that the free energy difference between the unstrained and strained states in the deoxy and mono-oxygenated states are Δg_0 and Δg_1, respectively, and that they have the following relation.

$$\Delta G_1^u - \Delta G_1^s = \Delta g_0 - \Delta g_1 \tag{13}$$

Δg_i can be regarded as the sum of the contributions from the heme (Δg_i^H) and the protein (Δg_i^P) moieties.

$$\Delta g_1 = \Delta g_1^H + \Delta g_1^P \tag{14a}$$

$$\Delta g_0 = \Delta g_0^H + \Delta g_0^P \tag{14b}$$

Δg_i^P arises from the changes of the protein tertiary and quaternary structures. As noted previously, the strained state results from the stronger intersubunit bonding and the concomitant intrasubunit hydrogen bonding, the protein moiety must be stabilized in the strained state ($\Delta g_i^P < 0$). In contrast, the heme moiety must be destabilized by the strain ($\Delta g_i^H > 0$). Since the change of the protein moiety causes destabilization of heme, the magnitudes of Δg_i^H and Δg_i^P would have some correlation. Accordingly, we assume a proportionality relation of their magnitudes.

$$|\Delta g_i^H| \propto |\Delta g_i^P| \tag{15}$$

Upon the change from the unstrained to the strained state, the frequencies of the porphyrin vibrations are only slightly altered (~ 2 cm^{-1} for the Raman lines around 1300–1650 cm^{-1}) with a linear correlation with the ν_{Fe-His} frequencies.[22]   The Fe–O_2 stretching frequencies of oxyHb[6,30] and the Fe—CO and C≡O stretching frequencies of carbonmonoxyHb[31] have practically no correlation with the oxygen affinity. Therefore, it is likely that the primary contribution of Δg_i^H arises from the Fe–His bond.

Δg_i^H contains the enthalpy and entropy terms. When the enthalpy changes are plotted against the entropy change, a linear relation is often found for a chemical equilibrium.[32]   Accordingly, Δg_0^H can be considered to be proportional to the enthalpy dif-

(30) Irwin, M. J.; Armstrong, R. S.; Wright, P. E. *FEBS Lett.* **1981**, *133*, 239–243.

(31) Rousseau, D. L.; Tan, S. L.; Ondrias, M. R.; Ogawa, S.; Noble, R. W. *Biochemistry* **1984**, *23*, 2857–2865.

(32) Iizuka, T.; Kotani, M. *Biochim. Biophys. Acta* **1969**, *181*, 275–286.

ference between the strained and unstrained states, and thus to the energy increase of the Fe–His bond (ΔV).

$$|\Delta g_0^H| \propto |\text{enthalpy change}| \propto |\Delta V| \qquad (16)$$

Equations 15 and 16 may correspond to assume that a change of protein upon the change of subunit interactions occurs in a concerted way that individual small changes in the protein[33] correlate linearly with the changes of the Fe–His bond in their sizes and also with the changes at the subunit interface.

Since we failed to observe the Fe–His stretching mode of mono-oxyHb, we have no idea about Δg_1^H at the present stage. However, note that the ν_{Fe-His} frequencies of the transient deoxyHb's, which occur within 10 ns of photolysis of COHb and are considered to have the ligated tertiary structure with a given quaternary structure, differ between the low- and high-affinity quaternary states[14,21a,b] quite similarly to the equilibrium deoxyHb's in the corresponding two states. Furthermore, most of the oxygen-binding curves could be well simulated by adjusting only one parameter of the three MWC parameters due to the presence of parameter correlation.[24,25] These facts may allow us to assume that Δg_1 is proportional to Δg_0.

$$\Delta g_0 \propto \Delta g_1 \qquad (17)$$

The three assumptions (eq 15–17) lead us to the proportionality between $\Delta g_0 - \Delta g_1$ and ΔV, that is

$$\Delta g_0 - \Delta g_1 = \kappa \Delta V \qquad (18)$$

where κ is simply a proportionality constant. Combining eq 18 and 13, we obtain

$$\Delta G_1^s = \Delta G_1^u + \kappa \Delta V \qquad (19)$$

We assume that ΔG_1^u is constant, because it is a free energy change upon oxygenation of an unstrained heme. Consequently

$$RT \ln K_1 = \gamma + \kappa \Delta V \qquad (20)$$

where γ is a constant. Substitution of ΔV from eq 20 into eq 11 yields

$$\Delta \nu = (V'''^2/2\mu k_s^2 \kappa)^{1/2} (RT \ln K_1 - \gamma)^{1/2} \qquad (21)$$

In accordance with eq 21, we fitted the observed points in Figure 5 to the equation

$$\nu = \nu_u - B(\log K_1 + C)^{1/2} \qquad (22)$$

and three parameters, ν_u, B, and C, were determined by the least-squares fitting[34] to the 24 observed points. The curve so calculated is drawn by a solid line in Figure 5. Despite the crude approximation the calculated curve well reproduces the observed points. This might imply the validity of the present treatment based on the strain model.

We defer discussion on the numerical values of parameters until the ν_{Fe-His} frequencies of the α and β subunits in the tetramer are determined separately. In fact, the frequency difference between the α and β subunits is significantly large in the low-affinity equilibrium state[35,36] as well as the transient state.[37] A point which awaits confirmation is whether the oxygen affinity of the α subunit in the tetramer is lower than that of the β subunit in accordance with the fact that the ν_{Fe-His} frequency of the α subunit is lower than that of the β subunit in the tetramer. However, it is stressed that the picosecond geminate recombination studies by Friedman et al.[23] showed a correlation between the relative yield of the 200-ps geminate process for O₂ and the ν_{Fe-His} frequencies of the transient. They also pointed out that the ν_{Fe-His} frequencies of the 10-ns transient deoxyHb are almost continuously distributed with some correlation with the ligand-binding reactivity.[38]

In conclusion, we wish to emphasize that, although the oxygen-binding curve of a single hemoglobin preparation under a certain solution condition can well be explained by the reversible transition between the strained and unstrained states, the strained state is not unique among a variety of hemoglobin preparations. As the magnitude of the strain depends on the solution conditions as well as the primary structure of the protein, it is a continuous variable. As a result, the ν_{Fe-His} frequencies are distributed continuously, and these values seem to have some correlation with the oxygen affinity (K_1) of hemoglobin in the deoxy state.

(33) Hopfield, J. J. *J. Mol. Biol.* **1973**, *77*, 207–222.

(34) The program LSANLDS in the library of NUCC (Nagoya University) made by Mr. Y. Hatano was used in this calculation. The authors are indebted to Mr. M. Saito of the computer center of this institute for his help upon running the program.

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