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Sequential Changes of the Fe–Histidine Bond upon Ligand Binding to Hemoglobin: Resonance Raman Study of α,α-Cross-Linked Co–Fe Hybrid Hemoglobins

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Abstract: Resonance Raman spectra were observed for α,α-cross-linked Co–Fe hybrid Hbs. The Fe–histidine (F8) stretching (ν_{Fe-His}), Fe–CO stretching (ν_{Fe-CO}), Fe–C–O bending (δ_{FeCO}), and O–O stretching (ν_{OO}) frequencies were determined for mono-, di-, and tri-Fe tetramers in a function of the number of bound ligands. The ν_{Fe-His} band center was determined with the accuracy of ±0.25 cm^{-1}, while uncertainties of the ν_{Fe-CO} and ν_{OO} frequencies were ±1 cm^{-1}. The ν_{Fe-His} band of α,α-cross-linked Hb appeared at 214 and 220 cm^{-1} for the equilibrium deoxy and CO-photodissociated transient forms (an average within 50 μs following photolysis), respectively, in good agreement with those of Hb A. The ν_{Fe-His} bands of mono- and di-α(Fe) tetramers were alike and were composed of two bands irrespective of the number of bound ligands, and they were significantly lower than those of mono- and di-(deoxy)β(Fe) subunits, which gave an intense symmetric band, indicating inequivalence between α and β subunits. This feature was qualitatively retained while the frequencies of two α(Fe) hemes and their relative intensities were slightly changed in the binding process of four ligands. In contrast, the ν_{Fe-His} frequency of the β subunits changed gradually as the number of bound ligands increased. The ν_{Fe-His} frequency of α(Fe) was lower by 4–6 cm^{-1} in the α,α- than in the β,β-cross-linked Co–Fe hybrid Hb, while those of β(Fe) were alike. The ν_{Fe-CO} frequency was higher for the α(Fe) than β(Fe) subunits by 2–4 cm^{-1} for both mono- and di-Fe species, and changed a little with the number of bound ligands for the α subunit but not for the β subunit. The ν_{OO} frequency of the oxyCo subunit was scarcely altered between the α and β subunits and also with the number of bound O_2 ligands. The present results about ν_{Fe-CO} and ν_{OO} bands were in agreement with the results previously obtained for the β,β-cross-linked Co–Fe hybrid Hb, suggesting that structural changes upon the increase in the number of the bound ligands are much larger in the proximal side than in the distal side. The behavior of the ν_{Fe-His} band suggests that the proximal structure little changes in the α subunit until binding of two ligands but that in the β subunit starts to change from the first ligand.

Introduction

Hemoglobin (Hb) has been extensively studied as an exemplary allosteric system,[1,2] and the structural mechanism involved in the sigmoidal behavior of the oxygen binding curves has been the subject of various spectroscopic studies (see ref 3 for a review). Although much experimental data can be explained by the concerted two-state model, which assumes the reversible transition between the high-affinity (R) and low-affinity (T) quaternary structures,[4] there are serious features which are incompatible with this theory.[5] An alternative model assumes sequential structural changes as the number of the bound ligands increases.[6] The data favorable to the latter were obtained from the ^{1}H NMR measurements of oxygenation process of Hb A.[7] In order to examine the validity of this model, isolation of partially ligated intermediate forms is indispensable, although practically it is extremely difficult with normal Hb A because of the presence of cooperativity.

Partially ligated intermediates have been investigated with three kinds of hybrid Hbs: (i) cyanomet valency hybrid Hbs which contain two CN-coordinated ferric hemes at either the α or β subunits and two deoxy hemes in the remaining ones;[8-13] (ii) M-type natural mutant Hbs;[14-16] (iii) metal-hybrid Hbs including Co–Fe,[17-21] Ru–Fe,[22] and Ni–Fe hybrid Hbs.[23] These compounds, which are all symmetric hybrids, are useful as models of

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intermediate di-ligated tetramers, but not other intermediates such as mono- and tri-ligated and asymmetric di-ligated species. The study of the asymmetrically ligated foms has been made possible since Walder et al.[24] succeeded in cross-linking the two lysine residues (Lys-82) of the β subunits by using bis(3,5-dibromosalicyl) fumarate. With this method, all of the mono-, di-, and tri-Fe metal-hybrid tetramers can be prepared,[21] and NMR studies of them[25] confirmed that the cross-linking does not alter the spectral characteristics of quaternary structures.

Resonance Raman (RR) scattering from the Fe–Co hybrid Hbs provides the Fe–histidine(F8) stretching ($\nu_{\text{Fe-His}}$) frequency of deoxyFe subunits, the O–O stretching (ν_{OO}) frequency of the oxyCo subunits, and the Fe–CO stretching ($\nu_{\text{Fe-CO}}$) and FeCO bending (δ_{FeCO}) frequencies of the CO-ligated Fe subunits (Fe$^{\text{CO}}$) by tuning the excitation wavelength between 400 and 440 nm.[20,26–28a] The $\nu_{\text{Fe-His}}$ frequency is known to distinguish between the T and R structures of the equilibrium[29] and CO-photodissociated transient deoxyHbs.[30] On the other hand, it has been demonstrated for natural mutant Hbs that the $\nu_{\text{Fe-His}}$ frequencies have a correlation with the oxygen affinities of individual species[31] and the continuous change of the $\nu_{\text{Fe-His}}$ frequency was interpreted satisfactorily in terms of the strain imposed on the Fe–His bond by globin.[32] The ν_{OO} frequency can reflect the hydrogen bonding to the bound oxygen[33] as well as to the proximal histidine[34] and the binding geometry of oxygen,[35] while the $\nu_{\text{Fe-CO}}$ frequencies are believed to provide information about geometrical distortion of the Fe–C–O group from the heme normal[16d,36,37] or electronic polarization of CO[38] and thus to document steric hindrance by nearby residues, or polarity of the environments near the bound CO.[39,40]

Our previous observations of the $\nu_{\text{Fe-His}}$ modes of the β,β-cross-linked Co–Fe hybrid Hbs[41] revealed that the binding of a ligand to a single β subunit causes a large structural change of the protein but that binding to a single α chain has much less effect, a result which is consistent with those of the NMR study.[25] Although these findings were inconsistent with the requirements of the two-state concerted models for oxygenation of Hb, it was necessary to examine whether these results were influenced by the β,β-cross-linking or not. Therefore, we prepared the α,α-cross-linked Fe–Co hybrid Hbs[42] and carried out RR experiments similar to those conducted for the β,β-cross-linked Hbs. Here we report the 406.7- and 441.6-nm excited RR spectra of the α,α-cross-linked Co–Fe hybrid Hbs with a specified number of ligands and discuss the difference between the α and β subunits during the oxygenation process on the basis of the $\nu_{\text{Fe-His}}$, ν_{OO}, $\nu_{\text{Fe-CO}}$, and δ_{FeCO} frequencies.

Experimental Procedures

Preparation of Samples. Hb A was purified from human blood by the method of Drabkin.[43] Hb C (β6-Glu→Lys) was separated and purified from hemolysates of human Hb S–Hb C blood by DE-cellulose column chromatography using a linear gradient of 30 mM Tris-HCl, pH 8.5, and 60 mM Tris-HCl, pH 7.7, under an atmosphere of CO; in practice, Hb C was eluted first around pH 8.4, followed by Hb S around pH 8.2 due to differences in their pI values.[44] CoHb A and CoHb C were prepared as reported,[45] and symmetric Co–Fe hybrid Hb A's were obtained according to the method of Ikeda-Saito et al.[17a] The preparation of α,α-cross-linked asymmetric Co–Fe hybrids was performed by a combination of the methods of asymmetric hybrid preparation[21a,25] and the α,α-cross-linking.[42a]

To prepare mono-Fe asymmetric Co–Fe hybrids, equimolar (approximately 2 mM metal) quantities of appropriate symmetric Co–Fe Hb A's and CoHb C were mixed in 0.1 M bis-Tris, pH 7.0, in a Thunberg tube and mixed with a 3-fold excess of IHP (inositol hexaphosphate). Oxygen was purged from the Thunberg tube with argon (purity 99.999%) with continuous stirring until complete deoxygenation of the mixture was achieved as judged by the visible absorption spectrum. A 1.3-fold excess of the cross-linking reagent [bis(3,5-dibromosalicyl) fumarate],[46] which was previously placed in the side arm, was then anaerobically added to the reaction mixture, followed by further purging with argon to ensure complete deoxygenation. The mixture was incubated at 37 °C for 2.5 h. The cross-linking reaction was stopped by adding a 100-fold excess of solid glycine. The resultant mixture was left standing on ice under an argon atmosphere for 1 h and then reoxygenated and concentrated using Centriflo ultrafiltration membrane cones (Amicon). The concentrate was applied onto a column of Ultrogel ACA44 (LKB), which was equilibrated with 0.1 M Tris, pH 7.2, and 1 M $MgCl_2$, in order to separate the cross-linked fraction from the unreacted one. The cross-linked fraction was eluted first and accounted for more than 50% of the initial material. The cross-linked fraction was passed through a column of G-25 equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and flushed with CO to minimize autoxidation of hemes during purification. The cross-linked fraction was loaded onto a column of CM-cellulose equilibrated with 10 mM potassium phosphate buffer, pH 6.7, and eluted with a linear gradient of 10 mM potassium phosphate buffer, pH 7.0, and 20 mM potassium phosphate buffer, pH 7.8. The three major fractions were eluted in the order of their electric charges; the fraction containing cross-linked Co–Fe hybrid Hb A was eluted first, followed by that of cross-linked, asymmetric Co–Fe Hb A–Hb C hybrid and that of cross-linked CoHb C. Therefore, the middle fraction contained the desired mono-Fe asymmetric Co–Fe hybrid Hbs. The same cross-linking procedure was applied to an equimolar mixture of appropriate symmetric Co–Fe Hb A and Hb C for preparation of tri-Fe asymmetric Co–Fe hybrid Hbs. The final preparations were assayed for purity by electrophoresis, isoelectric focusing (Gelman), and SDS-PAGE (sodium dodecyl sulfate-polyacry-

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lamide gel electrophoresis) (Phastsystem, Pharmacia).[47] All the preparations were concentrated to 3–5 mM (heme) and stored as fully oxy forms in a liquid nitrogen reservoir until ready for use.

Raman Measurements. The preparations were diluted to 60 μM (heme) with 100 mM potassium phosphate buffer, pH 7.0. The fully deoxy form was generated in a Raman cell by addition of a minimal amount of sodium dithionite and kept under a forepressure of 0.05 Torr of nitrogen during the Raman measurements. The carbonmonoxyFe/deoxyCo samples ($Fe^{CO}Co^{Deoxy}$) were obtained by flushing the fully deoxy preparation with CO gas, while the carbonmonoxyFe/oxyCo samples ($Fe^{CO}Co^{Oxy}$) were obtained by exposing the cell containing the $Fe^{CO}Co^{Deoxy}$ preparation to the CO and O_2 mixed gas with an appropriate ratio (CO:O_2 = 43:1 in volume[41]).

RR spectra were excited with either the 441.6-nm line of a He/Cd laser (Kinmon Electrics, CDR80MGE) or the 406.7-nm line of a Kr^+ ion laser (Spectra Physics, Stabilite 2016) and recorded with a JEOL-400D Raman spectrometer equipped with a cooled HTV-R649 photomultiplier. The Raman spectrometer was controlled by a computer (NEC, PC-9801). For the ν_{Fe-His} region, the spectral data were aquired at every 0.25 cm^{-1} and the peak position, read by the computer, was reproduced within ±0.25 cm^{-1} in the four measurements for all samples, although the sums of the four spectra are presented. However, for the ν_{Fe-CO} and ν_{OO} regions, the peak positions were read from the drawn spectra as usual and, therefore, the uncertainty of the peak position is ±1 cm^{-1}.

The fully deoxyHbs were measured by using an airtight quartz cuvette in the stationary state. The CO-photodissociated transient species were measured with an airtight quartz spinning cell (1800 rpm) containing 250–300 μL of the sample under tight focusing of the 441.6-nm irradiation (40 mW). Since CO is rebound during one turn of the cell (33 ms), the restored CO-bound form always meets the Raman excitation light. Under these conditions, the RR spectrum of the CO-bound form is observed when the laser power is low, whereas that of the photodissociated form is observed when the laser power is high. Since the diameters of the spinning cell and the excitation laser are ca. 20 mm and 100 μm, respectively, the transit time of a given molecule across the laser beam was about 50 μs. Therefore, the ν_{Fe-His} frequency of the photodissociated species means the average between 0 and 50 μs following photolysis. The population ratio of the photodissociated and non-photodissociated species was monitored by the intensity ratio of the ν_4 bands which appear around 1355 and 1375 cm^{-1} for the photodissociated and non-photodissociated species, respectively. The ν_{Fe-CO} band of $Fe^{CO}Co^{Deoxy}$ and $Fe^{CO}Co^{Oxy}$ species and the ν_{OO} band of $Fe^{CO}Co^{Oxy}$ forms were measured with the same spinning cell but the loose focusing of the 406.7-nm excitation light whose power was suppressed to 20 mW so as to prevent the Fe^{CO} heme from photodissociation as was evidenced by the lack of the 1355-cm^{-1} feature.

Methods for Determining the ν_{Fe-His} Frequency. As the RR spectrum in the ν_{Fe-His} region usually overlies on the tail of strong Rayleigh scattering, it is difficult to determine the center frequency of the ν_{Fe-His} band from the observed raw spectrum. To reproduce the shape of the Rayleigh wing as close as to that of the sample to be measured, Rayleigh scattering from α,α-cross-linked deoxyCoHb or oxyCoHb was measured when the Co subunit in the hybrid adopts the deoxy or oxy state, respectively. The difference between the sample spectrum and the corresponding Rayleigh scattering gives a background-free spectrum, which will be presented as the RR spectra in the ν_{Fe-His} region. The peak frequency was determined from the difference spectra with the aid of a computer. (The observed Raman spectra of the mono-α(Fe) deoxy tetramer, Rayleigh scattering from deoxyCoHb A, and their difference are given in Figure 1 of the supplementary material.)

Results

Figure 1 compares the RR spectra in the ν_{Fe-His} region of the α,α-cross-linked deoxyHb (lower) with those of native deoxyHb A (upper). Spectra A and A′ were obtained for the stationary deoxy state with the T structure, both yielding the ν_{Fe-His} band at 214 cm^{-1}. Spectra B and B′ were observed for CO-photodissociated species with a spinning cell. The ν_{Fe-His} frequencies of the transient species, which will be represented simply as ν_{Fe-His} of Fe^{CO} for convenience sake, are higher than those of the corresponding equilibrium deoxy states by similar amounts, although the α,α-cross-linked sample gives a lower frequency (by 1 cm^{-1}), presumably ascribable to a greater stabilization of the T structure. The bandwidths and spectral patterns are similar between A and A′ and between B and B′. This implies that the relaxation rate from the photodissociated transient to the equilibrium deoxy state is similar between B and B′. Accordingly, one can conclude that the Raman characteristics of the quaternary structure including its dynamics are unaltered by α,α-cross-linking.

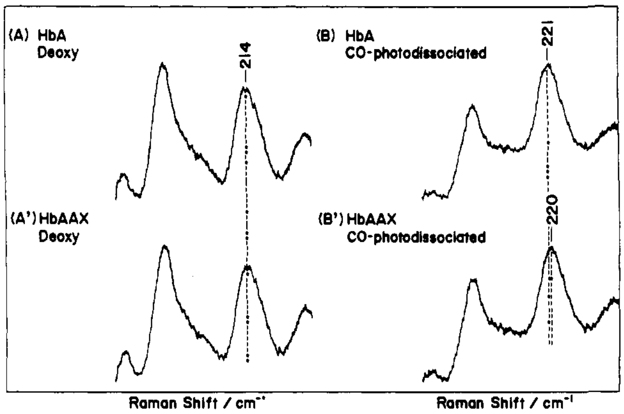

Figure 1. The RR spectra in the ν_{Fe-His} region of the equilibrium deoxy (left) and CO-photodissociated transient forms (right) of Hb A (upper) and α,α-cross-linked Hb (lower). Laser: 441.6 nm, 40 mW. The Rayleigh wing is corrected by subtracting the Rayleigh scattering observed for deoxyCoHb A from the observed Raman spectra. For measurements of spectra B and B′, a spinning cell (1800 rpm) was used.

The Co–Fe hybrid Hb is conveniently designated by the metal species (F for Fe and C for Co) of subunits in the order of $(\alpha\beta)_A$-$(\alpha\beta)_C$. For example, the di-α(Fe) species, $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_C$, is represented by FCFC AX (AX denotes α,α-cross-linking). The RR spectra in the ν_{Fe-His} region of mono- and di-Fe tetramers are shown in Figures 2 and 3, where A, B, and C represent the spectra for the equilibrium $Fe^{Deoxy}Co^{Deoxy}$, CO-photodissociated transient $Fe^{CO}Co^{Deoxy}$, and CO-photodissociated transient $Fe^{CO}Co^{Oxy}$ states, respectively. The number of bound ligands, which is denoted by L, is always 0 and 4 for A and C, respectively, but variable for B. The Fe hemes are contained in the α and β subunits for the samples of Figures 2 and 3, respectively.

As in the case of the β,β-cross-linked Co–Fe hybrid Hbs,[41] the ν_{Fe-His} bands of α(Fe) hybrids (Figure 2) are significantly broader than those of the β(Fe) hybrids (Figure 3) irrespective of mono- or di-Fe species, and it appears as if there are two bands. Therefore, two plausible band positions are labeled in each spectrum of Figure 2. These frequencies are appreciably lower than the corresponding frequencies of the β,β-cross-linked Co–Fe hybrid Hbs.[41] The presence of two bands for mono-α(Fe) means that there may be an equilibrium of two states within the single α subunit and that the Fe–His bond undergoes different magnitudes of strain in the two states. The intensity of the lower frequency component is stronger than that of the higher frequency component for the fully deoxy state of both FCCC AX and FCFC AX, and it is reversed for the fully ligated form. Such feature was also observed for the β,β-cross-linked Co–Fe hybrid Hbs.[41] It is noted for α(Fe) hemes that the frequency and shape of the ν_{Fe-His} bands remain unaltered among L = 0, 1, and 2, but they are appreciably altered for L = 4.

The ν_{Fe-His} band of the β(Fe) subunit is located at 216 cm^{-1} for the fully deoxy form (Figure 3A) and at 221 cm^{-1} for the fully ligated form (Figure 3C). The latter is the same frequency as that of normal COHb A (Figure 1B). It is clear that mono-Fe^{CO} and di-Fe^{CO} (Figure 3B) yield frequencies intermediate between those of the fully deoxy and fully ligated form and these frequencies indicate sequential changes as the number of the bound ligands increases.

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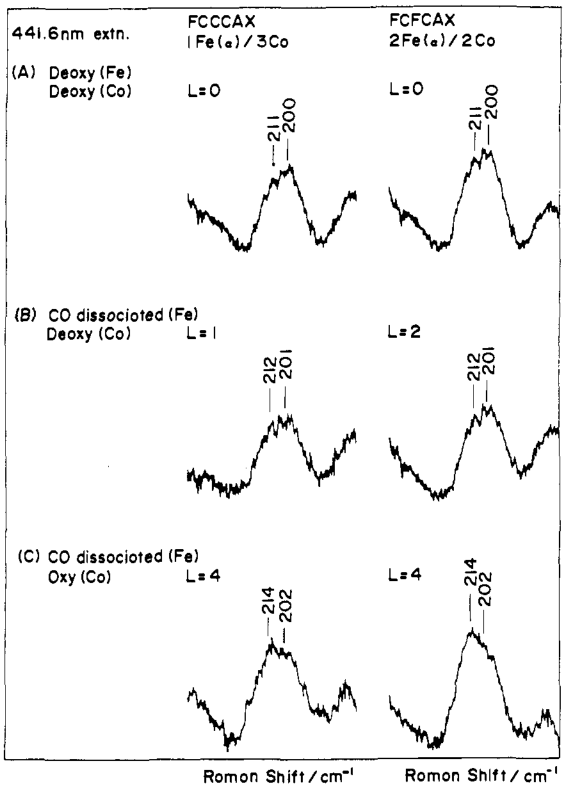

Figure 2. The RR spectra in the ν_{Fe-His} region of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_C$ (FCCC AX, left) and $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_C$ (FCFC AX, right): (A) $Fe^{Deoxy}Co^{Deoxy}$ species observed with a stationary cell, (B) CO-photodissociated transient form of $Fe^{CO}Co^{Deoxy}$ species observed with a spinning cell, (c) CO-photodissociated transient form of Fe^{CO}-Co^{Oxy} species observed with a spinning cell. Laser: 441.6 nm, 40 mW. The base line is corrected by subtracting the observed Rayleigh wing of the deoxy (A and B) or oxy form (C) of α,α-cross-linked CoHb.

Figure 4 shows the RR spectra in the ν_{Fe-His} region of tri-Fe hybrid Hbs. Since the intensity of the ν_{Fe-His} band of the β subunit is much stronger than that of the α subunit, the peak position is mainly determined by the ν_{Fe-His} band of the β subunit, although the contribution from the α subunit may lower the center of the composite band. Figure 4B gives the spectra of tri-ligated forms, $(Fe^{CO})_3(Co^{Deoxy})$, which are practically the same as those of fully ligated forms (Figure 4C), although there are slight differences between $[\alpha(Fe^{CO})\beta(Co)]_A[\alpha(Fe^{CO})\beta(Fe^{CO})]_C$ (218 cm^{-1}) and $[\alpha(Co)\beta(Fe^{CO})]_A[\alpha(Fe^{CO})\beta(Fe^{CO})]_C$ (220–221 cm^{-1}).

Figure 5 displays the 406.7-nm excited RR spectra in the ν_{Fe-CO} and δ_{FeCO} regions of $Fe^{CO}Co^{Deoxy}$ states of the mono-Fe (A and D), di-Fe (B and E), and tri-Fe (C and F) species. The ν_{Fe-CO} bands at 504–508 cm^{-1} are intense while the δ_{FeCO} bands around 583–586 cm^{-1} are broad and weak. Since the Co subunits are nonligated, the number of Fe subunits is equal to the number of bound ligands. The ν_{Fe-CO} frequencies are insensitive to the number of bound ligands. The difference between $\alpha(Fe^{CO})$ and $\beta(Fe^{CO})$ is most pronounced for the di-ligated form.

The same set of RR spectra as that in Figure 5 for the corresponding forms in which the Co subunit adopts the oxy state $(Fe^{CO}Co^{Oxy})$ is given in Figure 2 of the supplementary material. The ν_{Fe-CO} frequencies were little influenced by O_2 binding to the Co subunits, but again it was noticed that the $\alpha(Fe^{CO})$ subunits give higher ν_{Fe-CO} frequency than the $\beta(Fe^{CO})$ subunit, similar to Figure 5.

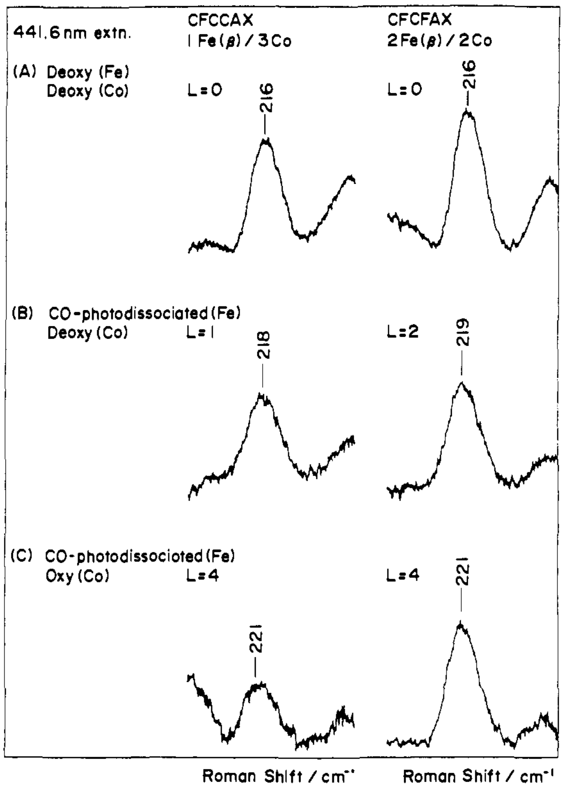

Figure 3. The RR spectra of $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_C$ (CFCC AX, left) and $[\alpha(Co)\beta(Fe)]_A$ $[\alpha(Co)\beta(Fe)]_C$ (CFCF AX, right) in the ν_{Fe-His} region. Implications of A–C and experimental conditions are the same as those in Figure 2.

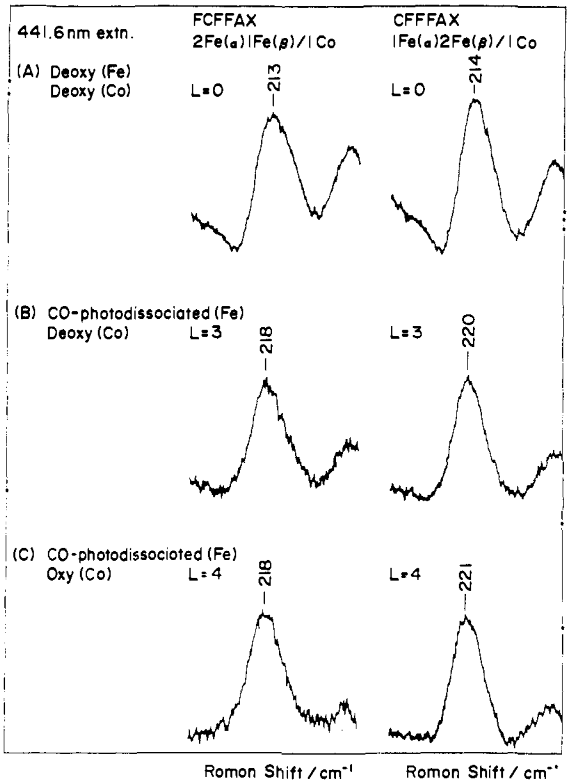

Figure 4. The RR spectra in the ν_{Fe-His} region of $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_C$ (FCFF AX, left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Fe)\beta(Fe)]_C$ (CFFF AX, right). Implications of A–C and experimental conditions are the same as those in Figure 2.

Figure 6 shows the 406.7-nm excited RR spectra in the ν_{OO} region of the $Fe^{CO}Co^{Oxy}$ combination of the mono-Fe (A and D), di-Fe (B and E), and tri-Fe (C and F) species. The ν_{OO} bands are seen at 1133–1134 cm^{-1} for all the species while their intensity increases with the number of the Co subunits. Similar to the results for the β,β-cross-linked Co–Fe hybrid Hbs, the ν_{OO} frequency scarcely differs between the α and β subunits and does not depend upon the number of bound O_2, although CO is bound to the Fe hemes and always $L = 4$ is attained.

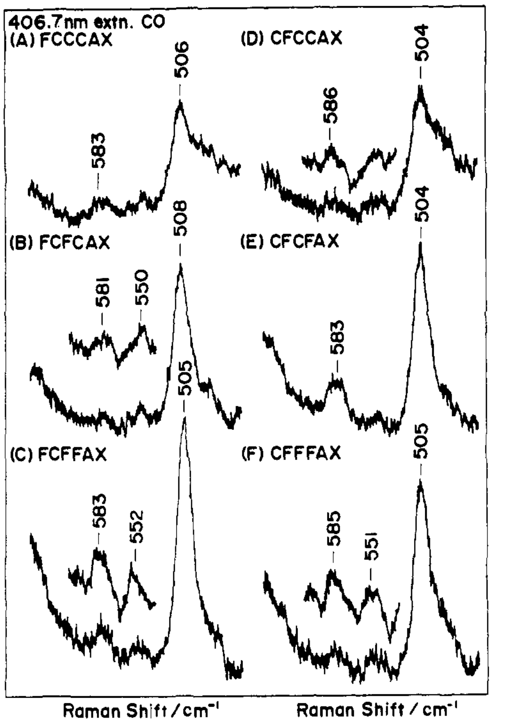

Figure 5. The RR spectra in the $\nu_{\text{Fe-CO}}$ region of α,α-cross-linked Co–Fe hybrid Hbs: (A) $[\alpha(\text{Fe})\beta(\text{Co})]_A[\alpha(\text{Co})\beta(\text{Co})]_C$, (B) $[\alpha(\text{Fe})\beta(\text{Co})]_A[\alpha(\text{Fe})\beta(\text{Co})]_C$, (C) $[\alpha(\text{Fe})\beta\text{Co})]_A[\alpha(\text{Fe})\beta(\text{Fe})]_C$, (D) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Co})\beta(\text{Co})]_C$, (E) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Co})\beta(\text{Fe})]_C$, (F) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Fe})\beta(\text{Fe})]_C$. Laser: 406.7 nm. The Co and Fe subunits adopt the deoxy and CO-bound forms, respectively.

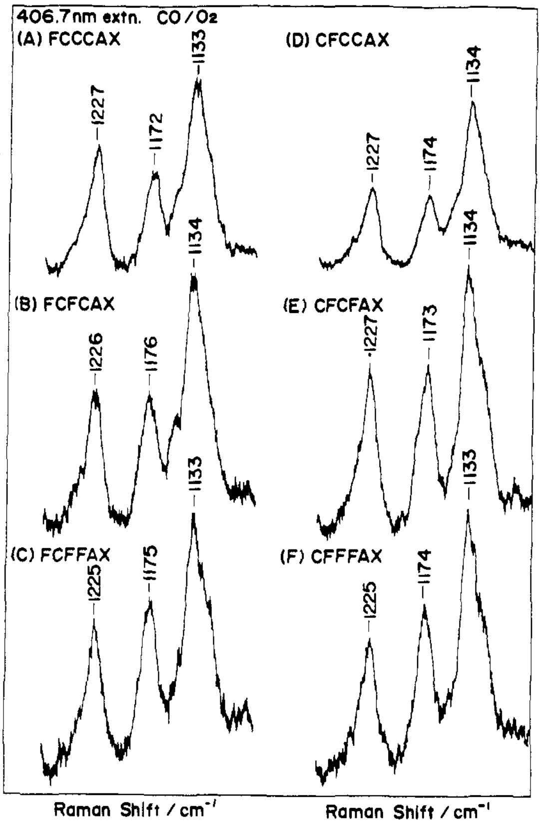

Figure 6. The RR spectra in the ν_{OO} region of α,α-cross-linked Co–Fe hybrid Hbs: (A) $[\alpha(\text{Fe})\beta(\text{Co})]_A[\alpha(\text{Co})\beta(\text{Co})]_C$, (B) $[\alpha(\text{Fe})\beta(\text{Co})]_A[\alpha(\text{Fe})\beta(\text{Co})]_C$, (C) $[\alpha(\text{Fe})\beta(\text{Co})]_A[\alpha(\text{Fe})\beta(\text{Fe})]_C$, (D) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Co})\beta(\text{Co})]_C$, (E) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Co})\beta(\text{Fe})]_C$, (F) $[\alpha(\text{Co})\beta(\text{Fe})]_A[\alpha(\text{Fe})\beta(\text{Fe})]_C$. Laser: 406.7 nm, 20 mW. The Fe and Co subunits adopt the CO- and O$_2$-bound forms, respectively.

Discussion

$\nu_{\text{Fe-His}}$ Band. The cross-linked metal hybrid Hb is currently the best material to explore the partially ligated intermediates of Hb. Shibayama et al.[48] demonstrated that the carefully purified β,β-cross-linked Hb exhibits the oxygen affinity, cooperativity, and an alkaline Bohr effect similar to those of unmodified Hb A. It is reported for the α,α-cross-linked Hb that the oxygen affinity is slightly lowered from that of Hb A while the cooperativity is unaltered.[49] Chatterjee et al.[42a] confirmed with X-ray crystallography that the α,α-cross-linking scarcely affects the protein structure except for the proximity of G3β-Glu to the cross-linking part, although the cross-linking fumarate may perturb the inter-subunit hydrogen bonds and salt bridges since it stays in the central cavity formed by the four subunits. The RR spectra of the α,α-cross-linked Hb were reported by Larsen et al.,[50] who confirmed that the $\nu_{\text{Fe-His}}$ frequencies of deoxy Hb and CO-photodissociated Hb are not influenced by the cross-linking. However, they were unable to examine the partially ligated intermediates because of the lack of metal hybrid Hbs.

The present study also confirmed for the tetra-Fe species that the $\nu_{\text{Fe-His}}$ frequency of the equilibrium deoxy form of the α,α-cross-linked Hb is almost the same as that of native Hb A (Figure 1A,A′). The $\nu_{\text{Fe-His}}$ frequencies of the CO-photodissociated transient species (Figure 1B,B′) are distinctly higher than that of the equilibrium deoxy species as expected from the kinetic Raman experiments, which located the $\nu_{\text{Fe-His}}$ band of the 10-ns transient around 222 (T)–230 (R) cm^{-1}.[30,51] It is emphasized that the frequencies and bandwidths of the transient $\nu_{\text{Fe-His}}$ bands of the native Hb A and α,α-cross-linked Hb are nearly the same. This means that the relaxation rate following CO-photodissociation is little altered by the cross-linking. Although $\nu_{\text{Fe-His}}$ = 221 cm^{-1} of the photodissociated species (Figure 1B) corresponds to the average frequency of molecules in the relaxation process within 50 μs following photolysis, we use hereafter this value as a standard of typical R structure, since the 50-μs average value is obtained commonly to all the CO-photodissociated species in this study.

The $\nu_{\text{Fe-His}}$ frequency reflects the magnitude of strain imposed on the Fe-His bond; as the strain becomes stronger, the Fe–His bond becomes weaker and the $\nu_{\text{Fe-His}}$ band shifts toward lower frequencies.[32] The broad feature of the $\nu_{\text{Fe-His}}$ RR bands for mono- and di-α(Fe) tetramers (Figure 2) compared with those for the corresponding β(Fe) tetramers (Figure 3) suggests the presence of two states within the same single α subunit in which the magnitude of the strain in the Fe–His bond differs. This is compatible with the previous EPR studies on symmetric[52] and β,β-cross-linked asymmetric[53] Fe–Co hybrid Hbs which indicate that the coordination in the α(Co) subunit(s) in the T-state tetramer is a pH-dependent equilibrium mixture of distorted (t) and undistorted (r) coordination states. Although EPR cannot tell the situation on the α(Fe) subunits, the present $\nu_{\text{Fe-His}}$ data on the α(Fe) subunit can be correlated to these EPR data to indicate that the heterogeneity exists within a single α subunit. There are several reports on the inequivalence between two α(Fe) subunits, and as will be discussed later and furthermore, Hori and Yonetani[54] observed the EPR inequivalence in the two

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α subunits of the $\alpha(\text{Co})_2\beta(\text{Fe})_2$ single crystal. This inhomogeneity within the α subunits in addition to that between α and β subunits is in conflict with the assumption of symmetry in the MWC model.[1]

In comparing the present $\nu_{\text{Fe-His}}$ frequencies shown in Figures 2–4 with those of the previous study on the β,β-cross-linked Hbs,[41] we note that the $\nu_{\text{Fe-His}}$ frequencies of the α subunits are lower in the α,α- than β,β-cross-linked Co–Fe hybrid Hbs by 4–6 cm^{-1}, suggesting that the Fe–His bond of the α subunit receives slightly larger strain in the α,α- than in the β,β-cross-linked hybrid Hbs. For the β,β-cross-linked Co–Fe Hbs,[41] mono-$\alpha(\text{Fe}^{\text{CO}})$ gave the same $\nu_{\text{Fe-His}}$ frequency as the fully deoxy species similar to Figure 2B (left), but di-$\alpha(\text{Fe}^{\text{CO}})$ gave the band close to that of the fully ligated form. This is in contrast with the result for the α,α-cross-linked Hb, shown by Figure 2B (right). The $\nu_{\text{Fe-His}}$ frequency of di-$\beta(\text{Fe}^{\text{CO}})$ (Figure 3B, right) is still lower than that of the fully ligated form (Figure 3C, right), while the corresponding two frequencies were the same for the β,β-cross-linked case.[41] These features may suggest that the α,α-cross-linking stabilizes the T structure slightly more than the β,β-cross-linking. In other words, the number of ligands necessary to completely change the quaternary structure from the deoxy-like to oxy-like structure is slightly larger for the α,α- than β,β-cross-linked Hb. This is compatible with the slightly larger strain, and thus lower O_2 affinity, in the α,α- compared to the β,β-cross-linked Hbs.

It was unexpected that the $\nu_{\text{Fe-His}}$ frequencies of the mono-$\alpha(\text{Fe})$ and di-$\alpha(\text{Fe})$ tetramers with oxyCo subunits (Figure 2C) were not completely altered to the R-type ones inasmuch as complete alteration was observed for the β,β-cross-linked species. The Raman intensity distribution of the $\nu_{\text{Fe-His}}$ band for mono-$\alpha(\text{Fe})$ species is changed between Co^{Deoxy} (Figure 2B) and Co^{Oxy} (Figure 2C) due to the quaternary structure change, and the same kind of change occurs between mono-$\alpha(\text{Fe})$ and di-$\alpha(\text{Fe})$ tetramer in Figure 2C despite that both are of $L = 4$. This means that the quaternary structure change to R is less complete in the α,α- than in the β,β-cross-linked Hbs. A similar trend is noted in the recent flash photolysis experiment of the $\alpha.\alpha$-cross-linked Hb.[50,55]

On the other hand, the $\nu_{\text{Fe-His}}$ frequencies of the mono-$\beta(\text{Fe})$ and di-$\beta(\text{Fe})$ tetramers in the fully deoxy state (Figure 3A) were the same as those in the β,β-cross-linked species.[41] Since the $\nu_{\text{Fe-His}}$ RR intensity of deoxy $\alpha(\text{Fe})_2\beta(\text{Fe})_2$ mainly comes from the β subunits due to the great difference in intensity between the α and β subunits,[56] the similarity in the $\nu_{\text{Fe-His}}$ RR band of deoxy state between native and α,α-cross-linked Hbs (Figure 1) presumably reflects the similarity in the β subunits of the two Hbs. Since the oxygen affinity of the β subunit not only is controlled by the Fe–His bond but also is influenced strongly by the orientations of distal residues, particularly E11-Val, the apparent absence of the difference in the $\nu_{\text{Fe-His}}$ frequencies of the β subunits between Hb A and α,α-cross-linked Hb does not necessarily imply that the oxygen affinity of the β subunits is unchanged by α,α-cross-linking. However, we note that the present results are consistent with the assumption that the oxygen affinity of the β subunits of the native and α,α-cross-linked Hbs are equal. In this case, the low oxygen affinity of the α,α-cross-linked Hb in comparison with that of native Hb A ($1/2$ in K_T and $1/10$ in K_R)[49] would be ascribed to the α subunit. The low oxygen affinity of the α subunits is consistent with the low $\nu_{\text{Fe-His}}$ frequency, that is, stronger strain imposed on the Fe–His bond by the globin.

In comparison of Figures 2 and 3, it is noted that the spectral difference between $L = 0$ and $L = 1$ or 2 is smaller for the $\alpha(\text{Fe})$ than for $\beta(\text{Fe})$ tetramer. The same behavior was also observed for the β,β-cross-linked hybrid Hbs and is confirmed to be an intrinsic property of Hb. This implies that the ligand binding to the β subunit more easily induces a quaternary structure change of the protein than does binding to the α subunit. The fact that the $\nu_{\text{Fe-His}}$ frequency of di-$\alpha(\text{Fe}^{\text{CO}})$ species ($L = 2$) is almost the same as that of $L = 0$ is consistent with the X-ray crystallographic results[57,58] which pointed out the similarity of the α heme structure as well as the $\alpha_1\beta_2$ interface structure of the α-di-ligated metal-hybrid Hbs to those of the T structure of native Hb A, while the Fe–His bond slightly differs between the α_1 and α_2 subunits.

It is noted for the α-ligated T-state Hb that there is no strain in the Fe–His bond of the β subunit while it exists in the α subunit,[59,60] and this is consistent with the present results shown in Figure 3B. In this regard, the α,α-cross-linked Co–Fe hybrid Hb seems to be much closer to native Hb A than a symmetric metal hybrid[17–20] or β,β-cross-linked Co–Fe hybrid Hbs.[21c,25,41] This might indicate more stabilization of the T than R structure by α,α-cross-linking as in a single crystal of deoxyHb A.[61] Without such a particular interaction, Co-substitution would have slightly destabilized the T structure, since the out-of-plane displacement of the Co^{II} ion is smaller than that of the Fe^{II} ion in the deoxy state.

For the tri-ligated state, the $\nu_{\text{Fe-His}}$ RR band of $[\alpha(\text{Fe}^{\text{CO}})\beta(\text{Co}^{\text{Deoxy}})]_A[\alpha(\text{Fe}^{\text{CO}})\beta(\text{Fe}^{\text{CO}})]_C$ (Figure 4B) is practically the same as that of the $L = 4$ state (Figure 4C), whereas that of $[\alpha(\text{Co}^{\text{Deoxy}})\beta(\text{Fe}^{\text{CO}})]_A[\alpha(\text{Fe}^{\text{CO}})\beta(\text{Fe}^{\text{CO}})]_C$ slightly changes upon binding of the last ligand to the α subunit. This implicates that the quaternary structure change has almost finished when three ligands are bound, while a single α-deoxy tetramer resists slightly more against the conversion toward the R structure than a single β-deoxy tetramer.

$\nu_{\text{Fe-CO}}$ Band. Yu et al.[36] pointed out that the $\nu_{\text{Fe-CO}}$ frequency reflects the distortion of the Fe–C–O geometry from the linear upright structure by the steric hindrance: 495 cm^{-1} for the linear upright to 514 cm^{-1} for 13° bending.[36a] The ab initio MO calculation by Augspurger et al.[38] suggested that the CO stretching frequency is affected by the electric field at the CO ligand, which is generated by the surrounding amino acid residues. This theory is compatible with the recent observation that the $\nu_{\text{Fe-CO}}$ frequencies of E7-mutant Mbs displayed a linear correlation with hydropathy indexes of E7 residues.[39] Mutation of the distal His of Hb also results in a change of the $\nu_{\text{Fe-CO}}$ frequency,[40] and its frequency depends on the polarity of the E7 residue rather than on its physical size. The present study reveals that the $\nu_{\text{Fe-CO}}$ frequency of the α subunit is higher than that of the β subunit by 2–4 cm^{-1}. This tendency was also observed for the β,β-cross-linked Co–Fe hybrid Hbs[41] and, therefore, is considered to be an intrinsic property of Hb. In the X-ray crystallographic analysis of deoxyHb with the T structure, the steric hindrance to CO would be larger in the β than in the α subunit due to the position of E11β-Val.[62] In the half-ligated metal hybrid (FeCO–Ni) Hb with the stabilized T structure,[63] the distance between the $C_{\gamma2}$ of E11β-Val and the O of bound CO is estimated to be 3.2 Å, which is shorter than that of normal COHb A (3.28 Å). Therefore, $\nu_{\text{Fe-CO}}$ should be the highest in the T-structure $\beta(\text{Fe}^{\text{CO}})$ heme, if the geometry solely determined the $\nu_{\text{Fe-CO}}$ frequency. This is contrary to the results shown in Figure 5. Presumably, the $\nu_{\text{Fe-CO}}$ frequency depends primarily on the polarity around CO (the more polar the surroundings are, the higher the $\nu_{\text{Fe-CO}}$ is), and its magnitude must be different between the α and β hemes. It

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is interesting that the effective polarity at the CO of $\beta(\mathrm{Fe^{CO}})$ is little altered (Figure 5D,E) upon ligand binding whereas that in the $\alpha(\mathrm{Fe^{CO}})$ is appreciably changed (Figure 5A,B). This is consistent with the note from the X-ray study[60] that the β heme is more loosely packed by a globin than the α heme.

The RR bands around 581–586 cm^{-1} in Figure 5 seem to be sensitive to the number of bound ligands, but we defer to discuss its implications until the assignment of this band is established; the band has been thought to arise from either the Fe–C–O bending fundamental,[36,37] its overtone,[64,65] or a combination,[66] and recently experimental evidences in favor of the last assignment are accumulating in this laboratory.

ν_{OO} Band. An oxygen-isotope-sensitive band was observed at 1133–1134 cm^{-1} for both α and β Co-substituted subunits. However, in IR spectra, three oxygen-isotope-sensitive bands are observed at 1155, ~1130, and 1106 cm^{-1}.[67] The ~1130-cm^{-1} IR band is reported to be broad[67] but presumably corresponds to the ν_{OO} mode as observed here, and the other two bands are probably the coupled modes.[28,34] X-ray crystallographic analysis of oxyHb[68] revealed that the hydrogen bond between the bound O_2 and distal His (E7-His) is weaker in the β than in the α subunit. Since the ν_{OO} RR band exhibited a small frequency shift between H_2O and D_2O solutions for O_2-CoHb,[33] the ν_{OO} frequency might be expected to be sensitive to the hydrogen bond in the distal side. In addition, the ν_{OO} frequency is sensitively altered by hydrogen bonding of the proximal histidine[28] and steric-induced distortions of the Fe–O–O geometry.[34] Nevertheless, the ν_{OO} frequencies of the $\alpha(\mathrm{Co^{Oxy}})$ and $\beta(\mathrm{Co^{Oxy}})$ hemes are the same as shown in Figure 6. This behavior is similar to the results obtained for the β,β-cross-linked Co–Fe hybrid Hbs.[41] This may suggest that the hydrogen bonding between bound O_2 and the distal His as well as the Fe–O–O geometry is not significantly different for the α and β cobalt-substituted subunits. The negligible changes of the ν_{OO} frequency with different numbers of bound O_2 are consistent with the X-ray crystallographic results which show that the geometry around E7α-His and bound O_2 remains unaltered between the R and T oxyHbs.[60] Recently, Mozzarelli et al.[69] found that O_2 is also bound to the β subunit of T-like oxyHb and stressed the difference between the α and β subunits. Parts D and E of Figure 6 in this study, which stand for the β subunits of tetra-ligated Hb with R structure, are very close to parts A and B, which stand for the α subunits.

In conclusion, the α,α-cross-linked Co–Fe hybrid Hb better stabilizes the T-like structure than does the β,β-cross-linked one and partially cancels the stabilization of the R-like structure by the Co-substitution. As a result, the α,α-cross-linked Co–Fe hybrid Hb seems to be a better model of the native Hb A than does the β,β-cross-linked one. The $\nu_{\mathrm{Fe-His}}$ frequency changes with the number of bound ligands, indicating sequential changes of the protein structure as the number of bound ligands increases. Differences between the two α subunits are prominent in the T-like structure, and the difference between the α and β subunits is quite large during every stage of ligand binding in the T structure. This is consistent with results of the NMR study which pursued the hyperfine shifted ^{1}H resonances specific to the α or β subunit of partially oxygenated intermediates of Hb A.[7] On the basis of the observed $\nu_{\mathrm{Fe-Co}}$ and ν_{OO} frequencies, the structural changes in the distal pocket during binding of ligands seem to be much smaller than those in the proximal side.

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Supplementary Material Available: Figures showing the method for determination of the $\nu_{\mathrm{Fe-His}}$ band and the RR spectra in the $\nu_{\mathrm{Fe-CO}}$ region of α,α-cross-linked Co–Fe hybrid Hbs (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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