Resonance Raman Spectra of Intermediate Ligated Forms of Hemoglobin: The ν_{Fe-His}, ν_{Fe-CO}, δ_{FeCO}, and ν_{OO} Modes of Cross-Linked Fe-Co Hybrid Hemoglobins

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Received July 19, 1988

Abstract: Asymmetrically Co-substituted Fe-Co hybrid hemoglobins were prepared by cross-linking Hb A to mutant Hb C (Glu β6→Lys) through bis(3,5-dibromosalicyl) fumarate, and their resonance Raman (RR) spectra were observed for various ligated forms. The Fe-histidine stretching (ν_{Fe-His}) band was distorted owing to overlapping with a tail of Rayleigh scattering, but its band position was determined with an accuracy of ± 1 cm^{-1} by subtracting the spectrum of the fully oxygenated form from each spectrum. With regard to the fully deoxy form, the ν_{Fe-His} mode gave an asymmetric band at 206 cm^{-1} with a shoulder at 215 cm^{-1} for the mono-α(Fe) tetramer, and it was intensified without a change of the band shape for the di-α(Fe) tetramer, while both mono- and di-β(Fe) tetramers yielded a symmetric and more intense band at 216 cm^{-1}. When three Co subunits were kept in the deoxy state, the photodissociated transient ν_{Fe-His} band of the carbonmonoxyFe (COFe) subunit of the mono-α(COFe) tetramer was observed at 206 cm^{-1} with a shoulder at 216 cm^{-1} similar to the fully deoxyHb, but that of the mono-β(COFe) tetramer was shifted to 220 cm^{-1}, and they were observed at 219 and 222 cm^{-1}, respectively, when three Co subunits were oxygenated. Accordingly, it was confirmed that the binding of a ligand to a single β subunit causes a large structural change of the protein, but that to a single α subunit has much less effect. The photodissociated transient ν_{Fe-His} frequencies of di-α(COFe) and di-β(COFe) tetramers were observed at 220-223 cm^{-1} which were unaltered by oxygenation to the remaining two Co subunits. This suggested that a structural change of the heme is almost completed upon binding of two ligands, in agreement with the oxygen binding properties. The Fe-CO stretching (ν_{Fe-CO}) and bending (δ_{FeCO}) frequencies were slightly lower and higher, respectively, for the β than for the α subunit for all ligation states. This implied that the anticipated steric hindrance by Val-Ellβ was released by an appropriate structural change of the globin. The OO stretching (ν_{OO}) frequency was the same between the α(Co) and β(Co) subunits for all the ligation states despite the fact that the hydrogen bonding of distal His to the bound oxygen is reported to be stronger for the α than for the β subunit.

Cooperativity of hemoglobin (Hb) has been a topic of extensive studies since it serves as a model of general allostery[1] and was explained by Perutz[2a,b] in terms of the concerted two-state model which assumes a reversible transition between the high-affinity (R) and low-affinity (T) quaternary structures. This theory reproduced satisfactorily a number of experimental data[3] for a first approximation, but there were other significant features which were inconsistent with this theory.[4a] The point to be clarified is whether some modification of the two-state model is adequate for describing the Hb cooperativity or a conceptionally different theory such as the sequential change model[5] is required. This question could be answered if the structures and functional properties of Hb were revealed as a function of the degree of oxygenation. However, because of the cooperative nature of oxygen binding, isolation of an intermediate-ligated state is not feasible by using normal Hb A.

Efforts to isolate intermediate-ligated states have been carried out so far with three kinds of compounds: (i) cyanomet valency hybrid Hbs which contain two CN-coordinated ferric hemes at the α or β subunits;[6-11] (ii) M-type mutant Hbs such as Hb M Boston, Hb M Iwate, Hb M Milwaukee, Hb M Hyde Park, and Hb M Saskatoon;[12,13] (iii) metal hybrid Hbs which contain the non-Fe metallo-protoporphyrin IX at the α or β subunits.[14a-d,15,16] In (i) and (ii), O_2 and CO can bind only to the Fe^{2+} heme but not to the $Fe^{3+}CN$ heme (i) or the abnormal met subunit (ii). In (iii) O_2 binds to the Fe^{2+} as well as Co^{2+} subunits and is not photodissociated with an ordinary power of laser light, but CO binds only to the Fe^{2+} subunit and can be photodissociated easily. Accordingly, with those compounds it was possible to isolate the di-ligated species of the tetramer and thus to characterize them by various spectroscopic, thermodynamic, and kinetic techniques. However, other intermediates including mono- and tri-ligated species and asymmetric di-ligated species could not be generated with such techniques.

A breakthrough was achieved by Miura and Ho[17] who succeeded in preparing asymmetric valency hybrid Hb's by utilizing a cross-linked reagent, bis(3,5-dibromosalicyl) fumarate, which had been known to link two lysine residues (Lys-82) of the two β subunits.[18] Inubushi et al.[19] applied the cross-linking technique

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to Co–Fe hybrid Hb and investigated the NMR spectra of mono-ligated states. With this method, all of the mono-Fe, di-Fe, and tri-Fe tetramers can be prepared and various ligated states can be generated. Inubushi et al.[19] confirmed that the cross-linking does not alter the NMR spectral characteristics of the proximal histidine and also of the quaternary structure, and found that when a single ligand was bound to the β subunit, a significant change occurred in the proximal histidine coordination of the remaining deoxy subunits with concomitant decrease in intensity of the T-state marker line, which arises from the proton of Tyr-42α hydrogen-bonded to Asp-99β at the $\alpha_1\beta_2$ interface, but no significant change took place upon binding of a single ligand to the α subunit. In those NMR studies the hyperfine-shifted resonances of the $N_\delta H$ proton of the proximal histidine (His) of the deoxyCo subunit can be monitored sensitively. However, it should be noted that the affinity constant for oxygen binding is greatly raised at the K_3 stage for the mono-COFe Co–Fe hybrid Hb's,[20] although it is raised at the K_4 stage for normal Hb A.[4b]

Resonance Raman (RR) scattering from the Fe–Co hybrid Hb's provides the Fe–His stretching frequency of the deoxyFe subunits, the OO stretching frequency of the oxyCo subunits, and the Fe–CO stretching and bending frequencies of the CO-ligated Fe subunits by tuning the laser wavelength between 400 and 440 nm.[15,21] Unfortunately, the Co–His stretching band of deoxyCoHb, the OO stretching band of oxyFeHb, and the Fe–His stretching band of oxyFeHb have not been confidently identified yet. It is emphasized, however, that the Fe–His stretching mode (ν_{Fe-His}) of deoxyFeHb distinguishes between the two quaternary structures[22] and reveals the difference between the α and β subunits.[11,15] Furthermore, the ν_{Fe-His} frequency was demonstrated to change continuously in accord with a change of the oxygen affinity,[23a] and the frequency change was interpreted on the basis of the strain imposed on the Fe–His bond by globin.[23b] The OO stretching mode (ν_{OO}) can reflect hydrogen bonding to the bound oxygen[24] and binding geometry.[25] The Fe–CO stretching (ν_{Fe-CO}) and bending (δ_{FeCO}) frequencies are reported to serve as a structural probe of the heme pocket.[26a,b] Accordingly, simultaneous measurements of these frequencies for specified intermediates are expected to bring about new structural information on the Hb cooperativity. Here we report the 406.7- and 441.6-nm excited RR spectra of the cross-linked Fe–Co 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 ν_{Fe-His}, ν_{OO}, ν_{Fe-CO}, and δ_{FeCO} frequencies.

Experimental Procedures

Materials. The cross-link was formed between ($\alpha\beta$) dimers of Hb A and Hb C (Glu β6→Lys) because Hb C has a larger positive charge than Hb A, which enabled us to distinguish the $(\alpha\beta)_A(\alpha\beta)_C$XL tetramer (XL denotes "cross-linking of the parenthesized units", and subscripts A and C represent the subunits of Hb A and Hb C, respectively) from $(\alpha\beta)_A$-$(\alpha\beta)_A$XL and $(\alpha\beta)_C(\alpha\beta)_C$XL by ion-exchange chromatography. The cross-linking reaction was carried out for 4 h at 0 °C with bis(3,5-dibromosalicyl) fumarate which was synthesized according to Walder et al.[18] The unreacted Hb's were separated from the cross-linked molecules by gel filtration with the column of Ultrogel AcA44 (LKB) according to Miura and Ho.[17] Separation of the three components of the cross-linked fraction was achieved on a CM-cellulose column (2 × 15 cm) at 4 °C equilibrated with 0.01 M phosphate buffer at pH 6.7 to which a linear gradient of 0.015 M phosphate buffer at pH 6.7 and 0.020 M phosphate at pH 8.0 was applied. Hb A and Hb C were purified from expired human blood and homozygous CC blood, respectively, by the method of Drabkin.[27] Cobalt substitutions of Hb A and Hb C were carried out as described previously.[14a,28] All samples with concentration 3–5 mM (metal) were stored in liquid nitrogen until ready for use and diluted to a concentration of 60 μM (metal) by 100 mM potassium phosphate buffer pH 7.0 for the measurements.

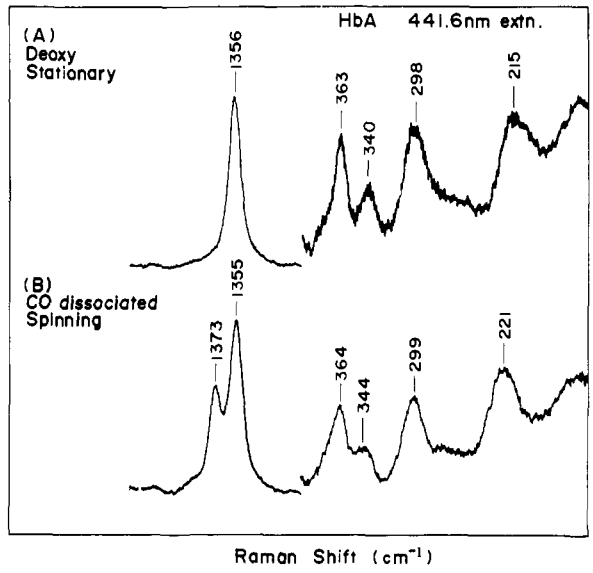

Figure 1. 441.6-nm excited resonance Raman spectra of normal Hb in the ν_4 and ν_{Fe-His} band regions: (A) deoxy state observed with the stationary cell, (B) photodissociated transient state of carbonmonoxyHb observed with a spinning cell (1800 rpm) (laser power 22 mW).

Fully deoxy samples were prepared by addition of a minimal amount of sodium dithionite and kept under a fore-pressure of 0.05 Torr of air during the Raman measurements. The carbonmonoxyFe/deoxyCo samples ($Fe^{CO}Co^{deoxy}$) were obtained by flushing with CO gas into the fully deoxy preparation, while the carbonmonoxyFe/oxyCo samples ($Fe^{CO}Co^{oxy}$) were obtained by putting the CO and O_2 mixed gas with an appropriate ratio (CO:O_2 = 43:1 in volume) into the cell containing the $Fe^{CO}Co^{deoxy}$.

Methods. RR spectra were excited by the 441.6-nm line of a He/Cd laser (Kinmon Electrics, Model CDR80MGE) and the 406.7-nm line of a Kr ion laser (Spectra Physics, Model 164) and measured with a JEOL-400D Raman spectrometer equipped with an RCA-31034a photomultiplier. Raman shifts were calibrated with indene (500–1600 cm^{-1}) and CCl_4 (200–500 cm^{-1}) and determined with an accuracy of 1 cm^{-1}.

The fully deoxy state was measured with an air-tight stationary cell. The CO-bound forms of $Fe^{CO}Co^{deoxy}$ and $Fe^{CO}Co^{oxy}$ were measured with an air-tight spinning cell (1800 rpm) by exciting it with a weak laser power of the 406.7-nm line. Their photodissociated transient forms were measured with the same cell but with a higher power of the 441.6-nm line (80 mW). In this method, the CO-bound form is restored after photodissociation, if it occurred, within one turn of the cell (33 ms). Only when a molecule is photodissociated by the first photon, and Raman scattering is excited by the second photon in a single exposure, can the ν_{Fe-His} band be observed. When the first photon gives rise to Raman scattering, its spectrum should be of the CO-bound form which never yields the ν_{Fe-His} RR band upon excitation at 441.6 nm. The population ratio of the photodissociated and nondissociated forms can be monitored by the ν_4 band which should appear at ~1355 and ~1375 cm^{-1} for the former and latter, respectively. Since the diameters of the cell and the laser beam were ca. 2 cm and 100 μm, respectively, the transit time of a given molecule across the laser beam was about 50 μs. Therefore, the observed ν_{Fe-His} RR band corresponds to a sum of the contributions from transient species present within 50 μs after photodissociation.

Results

It is known that the ν_{Fe-His} frequency of the photodissociated transient carbonmonoxyHb (COHb) is distinctly higher than that of deoxyHb in equilibrium[29a–c] and differs among various Hb's

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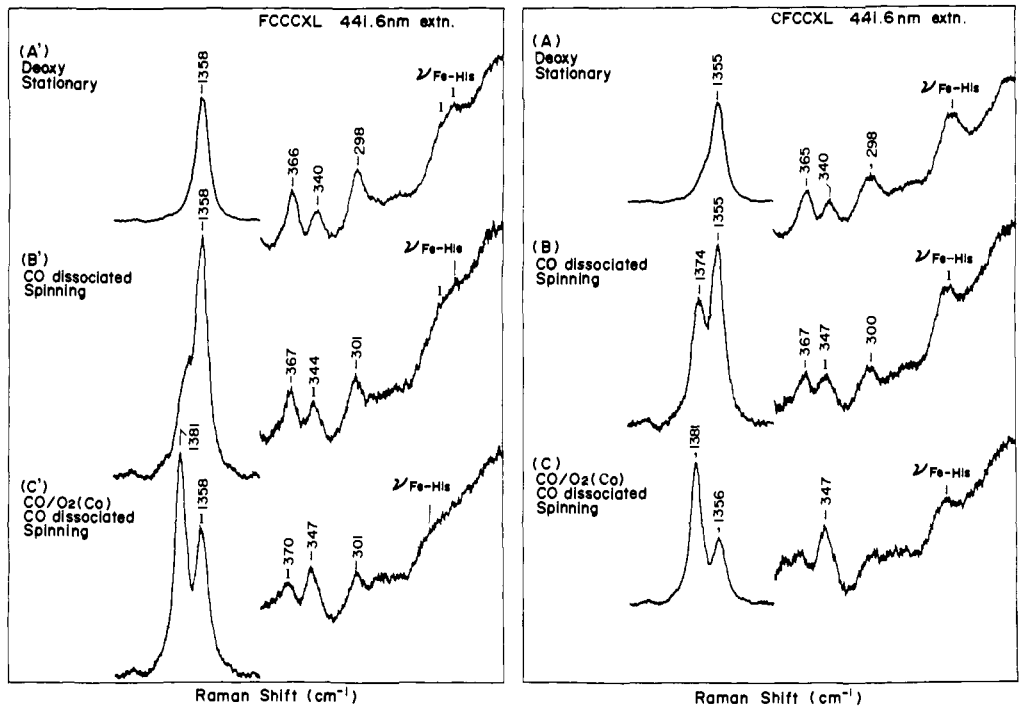

Figure 2. 441.6-nm excited resonance Raman spectra of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ (FCCCXL, left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$ (CFCCXL, right) in the ν_4 and ν_{Fe-His} regions: (A and A') $Fe^{deoxy}Co^{deoxy}$ combination observed with a stationary cell; (B and B') Co^{deoxy} and photodissociated transient Fe^{deoxy} combination observed with the spinning cell; (C and C') Co^{oxy} and photodissociated transient Fe^{deoxy} combination observed with the spinning cell (laser power, 22 mW).

with different degrees of oxygen affinity.[30a,b] The transient ν_{Fe-His} frequencies might reflect the Fe–His bonding in the ligated state. Accordingly, we observed such transient species in addition to the equilibrium ones. Since the ν_{Fe-His} frequency of the transient species is a function of time after photodissociation,[29] it is important to know what frequency is obtained for normal Hb A with the present instrumental and laser conditions. Figure 1 compares the RR spectrum of the photodissociated transient species (B) with that of the equilibrium deoxyHb (A). Although, the 1373-cm^{-1} band in spectrum B indicates the presence of an appreciable amount of ligated Hb, the 221-cm^{-1} band in spectrum B is assigned exclusively to the ν_{Fe-His} mode of the photodissociated transient species, since the ligated species do not give rise to the ν_{Fe-His} RR band. This frequency is distinctly higher than that of the equilibrium deoxyHb at 215 cm^{-1} and close to that reported for the 0.3-μs photoproduct of COHb.[29b] The spectral difference between A and B in the 340-cm^{-1} region resembles that reported by Friedman et al.[29a] Since it became confident that the spinning cell technique provides the ν_{Fe-His} band of the photodissociated transient COHb, we observed the ν_{Fe-His} band of the cross-linked Fe–Co hybrid Hb's for three different species: (A) equilibrium deoxyFe subunit with deoxyCo subunits, (B) photodissociated transient COFe subunit with deoxyCo subunits, (C) photodissociated transient COFe subunit with oxyCo subunits.

Figure 2 shows the RR spectra in the ν_{Fe-His} region of mono-Fe tetramers: $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ (left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$ (right). In order to monitor the extent of photodissociation, the RR spectra in the ν_4 region[31] is also depicted in Figure 2. The 1355–1358-cm^{-1} band stands for the deoxyFe subunits while the bands at 1374 and 1381 cm^{-1} arise from the nonphotodissociated COFe subunit and the oxyCo subunits, respectively. The ν_{Fe-His} band, which appears around 200–220 cm^{-1}, is broader and weaker for the α(Fe) than β(Fe) subunit as previously pointed out for the symmetric Fe–Co hybrid Hb's.[11,15] Since this is true with the tetramer having a single Fe subunit, the broad bandwidth could not be ascribed to possible difference in the ν_{Fe-His} frequencies between the two α subunits. The relative intensity of the 347-cm^{-1} band to the others increases in spectra C and C', but it is due to the contribution from the oxyCo subunit.

Figure 3 shows the RR spectra in the ν_{Fe-His} regions of di-Fe tetramers: $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_CXL$ (left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Fe)]_CXL$ (right). The spectra of the α(Fe) (A') and β(Fe) (A) subunits of the di-Fe tetramers in the equilibrium deoxy state resemble those of the mono-α(Fe) and mono-β(Fe) subunits shown in Figure 2, indicating that the spectra of two α(Fe) or two β(Fe) subunits are mutually similar. The broad feature of the ν_{Fe-His} bands in Figures 2 and 3 did not allow us to determine their band centers unequivocally. Since the fully oxygenated forms of each species give rise to the background due to the tail of Rayleigh scattering similar to the species in question but do not yield the ν_{Fe-His} band, we subtracted the spectra of the fully oxygenated form of the corresponding species indicated in Figures 2 and 3 from each spectrum, and the resultant difference spectra in the ν_{Fe-His} region are depicted in Figure 4, where the left and right panels represent the spectra of the α(Fe) and β(Fe) subunits, respectively, and the left and right sides in each panel represent the spectra of mono- and di-Fe substituted hybrids, respectively. Since the oxy- and deoxyCo porphyrins have a clear band around 260 cm^{-1}, but the equilibrium deoxyFe porphyrin has a weaker and broad feature there, a negative band becomes stronger in the difference spectra as the number of the equilibrium deoxyFe subunits decreases. Therefore, for convenience, assumed base lines are drawn by broken lines in individual spectra. When the coefficient in the difference calculations was altered within a reasonable range, the peak frequency of the ν_{Fe-His} band remained unaltered within 1 cm^{-1}.

The ν_{Fe-His} frequency is distinctly lower for the α than β subunit in the equilibrium deoxy state (Figure 4, spectra A and A'), but this difference becomes much smaller when three or two ligands are bound to the tetramer (C and C'). This is consistent with the fact that the frequency difference between the ν_{Fe-His} mode of the α and β subunits is diminished in the R state tetramer.[11,15] In

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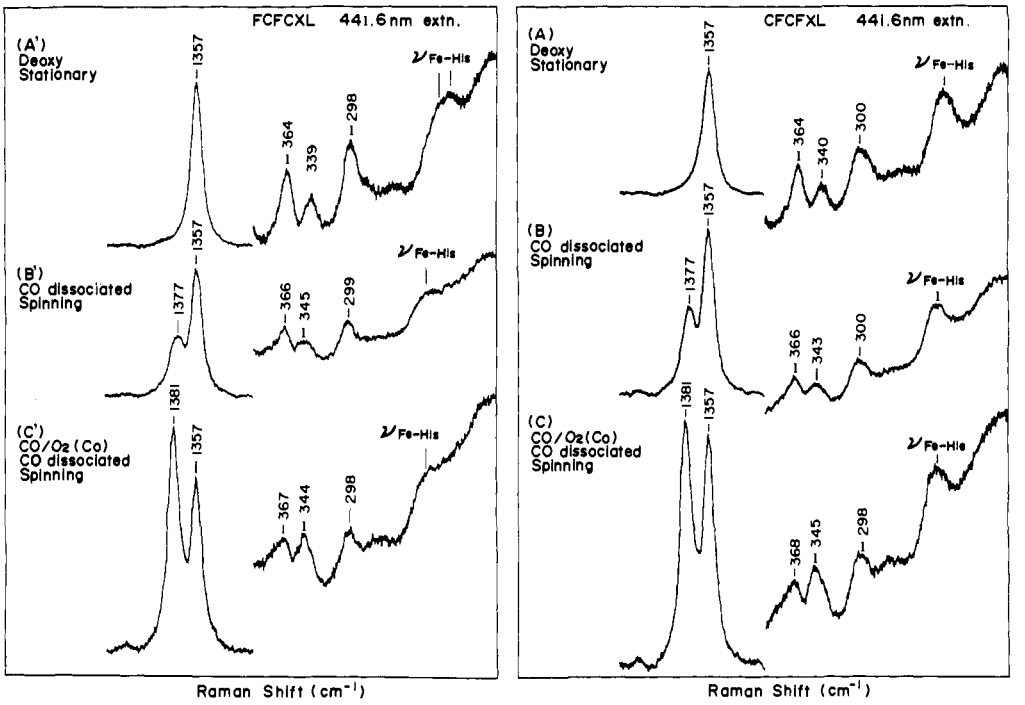

Figure 3. 441.6-nm excited resonance Raman spectra of $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_CXL$ (FCFCXL, left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Fe)]_CXL$ (CFCFXL, right) in the ν_4 and ν_{Fe-His} regions. The implications of characters (A–C and A′–C′) are the same as those in Figure 2.

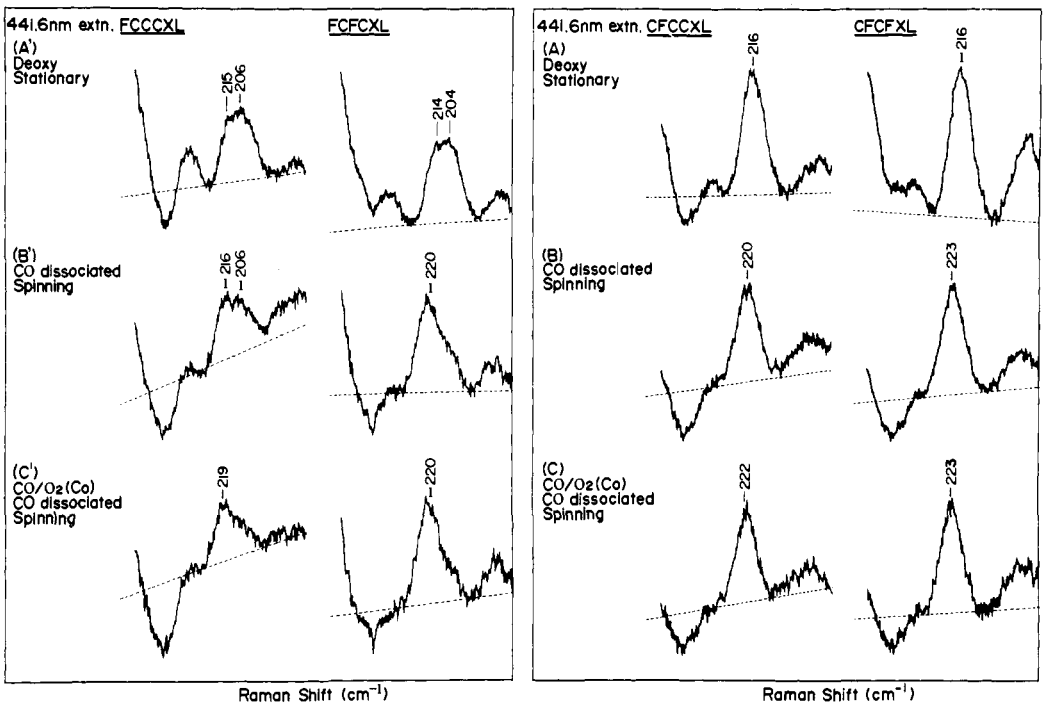

Figure 4. The base-line corrected Fe–His stretching bands of the Fe–Co hybrid Hb's. The spectrum of fully oxygenated form of each compound was subtracted from the corresponding spectra shown in Figures 2 and 3. The implications of characters (A–C and A′–C′) are the same as those in Figure 2. The spectra shown in the left and right sides of both panels were obtained from Figures 2 and 3, respectively. Abbreviations for specifying compounds are explained in the captions of Figures 2 and 3. Broken lines indicate the base line in the difference spectra.

the mono-ligated tetramer, the photodissociated transient ν_{Fe-His} frequency of the α(COFe) subunit remains at the frequency of the equilibrium deoxyHb (Figure 4B′). However, it is stressed that the photodissociated transient ν_{Fe-His} frequency of the β subunit is appreciably raised (Figure 4B) and the resultant frequency is intermediate between the two typical values of the fully deoxy and fully ligated states. When two ligands are bound to the tetramer, the difference between the photodissociated transient ν_{Fe-His} frequencies of the α(COFe) and β(COFe) subunits noticeably reduces in spectra B and B′ as well as C and C′ in Figure 4. Furthermore, the ν_{Fe-His} frequencies of the photodissociated transient COFe subunits are unaltered by binding of oxygen to the remaining two Co subunits. These facts imply that the tetramer adopts the R structure when two ligands are bound and this is irrespective of whether they are bound to the α or β subunits.

Figure 5 shows the RR spectra in the ν_{Fe-His} and ν_4 regions of tri-Fe tetramers: $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (right). The center frequency of the ν_{Fe-His} band was determined in the same way as those in Figure 4, although the apparent peak positions looked lower than that cited owing to the contribution from the Rayleigh tail. It is confirmed again that the photodissociated transient ν_{Fe-His}

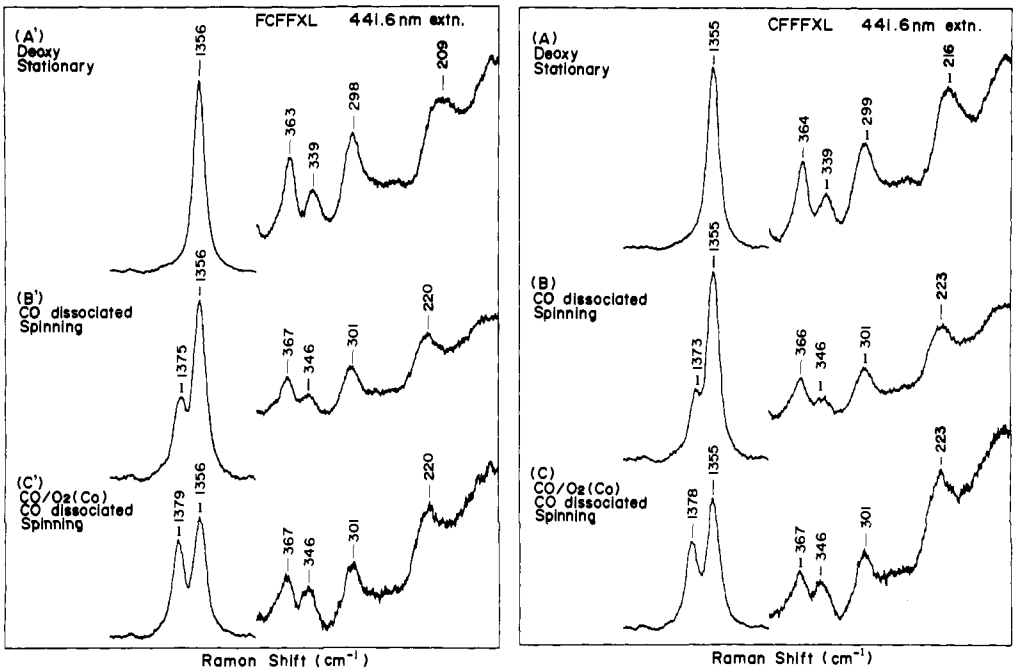

Figure 5. 441.6-nm excited resonance Raman spectra of $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (FCFFXL, left) and $[\alpha(Co)\beta(Fe)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (CFFFXL, right) in the ν_4 and ν_{Fe-His} regions. The band centers of the ν_{Fe-His} bands were determined from the difference spectra similar to those shown in Figure 4. The implications of characters (A–C and A′–C′) are the same as those in Figure 2.

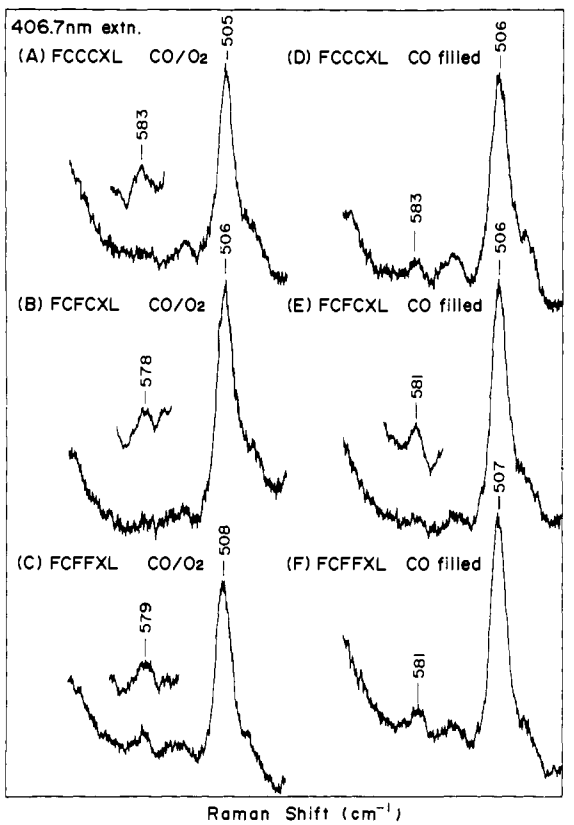

Figure 6. 406.7-nm excited resonance Raman spectra in the ν_{Fe-CO} and δ_{FeCO} regions of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ (A and D), $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_CXL$ (B and E), and $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (C and F). The Co subunits adopt the oxygenated form in A–C and the deoxygenated form in D–F, while the Fe subunits adopt the CO-bound form.

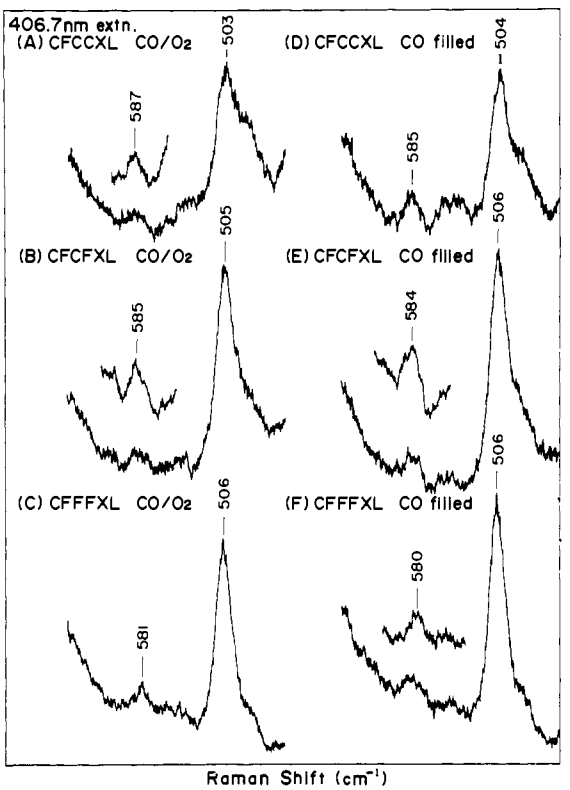

Figure 7. 406.7-nm excited resonance Raman spectra in the ν_{Fe-CO} and δ_{FeCO} regions of $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$ (A and D), $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Fe)]_CXL$ (B and E), and $[\alpha(Co)\beta(Fe)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (C and F). The Co subunits adopt the oxygenated form in A–C and deoxygenated form in D–F while the Fe subunit adopt the CO-bound form.

frequency is no longer affected by the presence of the ligand in a single Co subunit and is essentially the same as that of normal Hb A shown in Figure 1B.

Figure 6 shows the RR spectra in the ν_{Fe-CO} region of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ (A, D), $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_CXL$ (B, E), and $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_CXL$ (C, F) in which the Co subunits are oxygenated in A, B, and C and deoxygenated in D, E, and F. The ν_{Fe-CO} and δ_{FeCO} bands appear at 505–508 and 578–583 cm^{-1}, respectively, although the latter might be an overtone of δ_{FeCO}.[32] The results for the reversed

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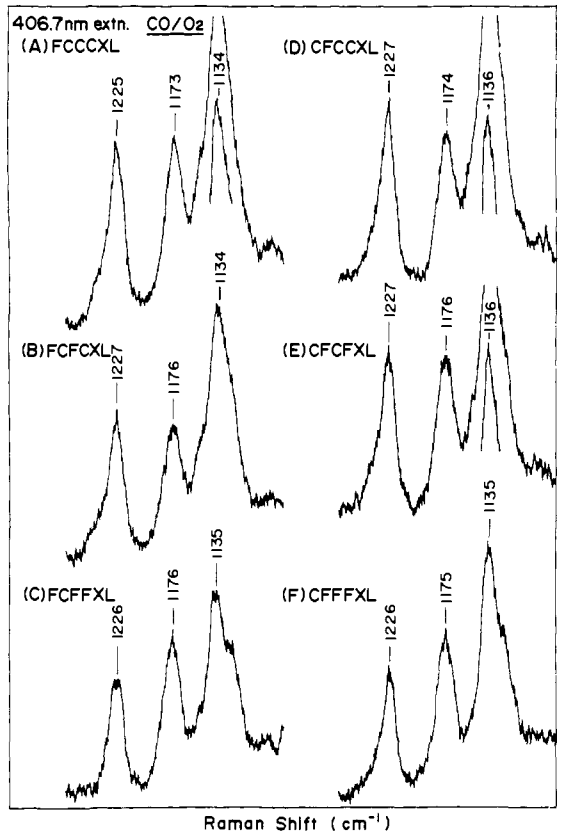

Figure 8. 406.7-nm excited resonance Raman spectra in the ν_{OO} region of the Fe–Co hybrid Hb's with $Fe^{CO}Co^{oxy}$ combination. The notation of compounds is explained in the caption of Figure 2 (laser power 6–9 mW).

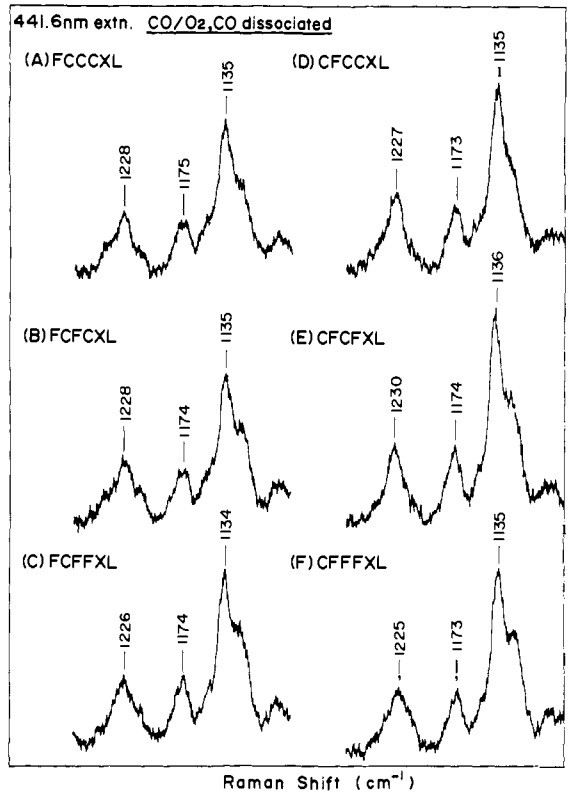

Figure 9. 441.6-nm excited resonance Raman spectra in the ν_{OO} region of the Fe–Co hybrid Hb's with the Co^{oxy} and photodissociated transient Fe^{deoxy} combination. These spectra were obtained for the same samples and under the same conditions as those for C and C′ in Figures 2, 3, and 5.

combination of the metal ions in the two subunits are summarized in Figure 7, where spectra A–F stand for the same ligation conditions of the Co subunits as those in Figure 6. Comparison of Figure 6 with Figure 7 indicates that the RR band due to the ν_{FeCO} mode is weaker and therefore becomes more asymmetric due to relatively increased contribution from a nearby porphyrin band. The ν_{FeCO} frequency is lower for the β(COFe) subunit (Figure 7) than for the α(COFe) subunit (Figure 6). This is little affected by binding of oxygen to the remaining Co subunits. On the other hand, the δ_{FeCO} (or $2\delta_{FeCO}$) frequencies are generally higher for the β(COFe) subunit (Figure 7) than for the α(COFe) subunit (Figure 6). Accordingly, the lower the ν_{Fe-CO} frequency is, the higher the δ_{FeCO} frequency is. It is noticed that as the number of the bound CO molecules increases, the ν_{Fe-CO} frequency is slightly raised.

Tsubaki and Yu[21] demonstrated, by the $^{16}O_2 - {}^{18}O_2$ isotope substitutions, that the dioxygen stretching mode of the bound oxygen of oxyCoHb, located near 1130 cm^{-1}, was resonance enhanced on excitation at 406.7 nm. Accordingly, we investigated the OO stretching RR band of the cross-linked Fe–Co hybrid Hb's with the 406.7-nm excitation line. Figure 8 shows the RR spectra in the ν_{OO} region of the oxy forms of $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_C$XL (A), $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Co)]_C$XL (B), $[\alpha(Fe)\beta(Co)]_A[\alpha(Fe)\beta(Fe)]_C$XL (C), $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_C$XL (D), $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Fe)]_C$XL (E), and $[\alpha(Co)\beta(Fe)]_A[\alpha(Fe)\beta(Fe)]_C$XL (F), in which the Fe subunits are always the CO-bound form. The ν_{OO} bands of the mono-α(Co) (F) and mono-β(Co) subunits (C) are located at 1135 cm^{-1}. The intensity of these bands increases upon an increase of the number of the oxy-Co subunits, but their frequencies change little. We emphasize that the ν_{OO} frequency is essentially the same between the α and β subunits.

Figure 9 shows the 441.6-nm excited RR spectra in the ν_{OO} region of the same compounds as those in Figure 8 except that the Fe subunits adopt the photodissociated transient state. The RR spectra in the ν_{Fe-His} and ν_4 regions of these compounds were shown by spectra C and C′ in Figures 2, 3, and 5, and, therefore, it is apparent that CO is photodissociated but O_2 remains in the Co subunits. Since the ν_{OO} band is less intensity-enhanced at this excitation wavelength and there are doublet bands at 1135 and 1116 cm^{-1} for the deoxyFe subunit, the contribution of the ν_{OO} mode to the 1134–1135-cm^{-1} bands in Figure 9 is not always large. However, its contribution is appreciable because the ratio of the peak intensity at 1135 cm^{-1} to that at 1116 cm^{-1} increases as the increase of the number of the Co subunits.

Discussion

Fe–Histidine Stretching Mode. The asymmetry of the ν_{Fe-His} RR band of deoxyHb A (Figure 1A) was interpreted in terms of the difference between the ν_{Fe-His} frequencies of the α and β subunits by using the valency hybrid Hb's first[11] and was later confirmed with the Fe–Co hybrid Hb's.[15] The latter study pointed out that the ν_{Fe-His} RR band was significantly asymmetric for $\alpha(Fe)_2\beta(Co)_2$ in comparison with that for $\alpha(Co)_2\beta(Fe)_2$ and suggested a possibility of heterogeneity of two α subunits. A powder ESR study with the Q band resonator indicated the presence of two types of Co(II) paramagnetic centers which are in a quaternary structure-sensitive equilibrium for deoxy α-$(Co)_2\beta(Fe)_2$.[33a] The presence of some difference between two α(Co) subunits was also noted from the ESR study[33b] for a single crystal of $\alpha(Co)_2\beta(Fe)_2$ deoxyHb. However, the ν_{Fe-His} RR band of the tetramer with a single α(Fe) subunit shown in Figure 2A′ exhibited asymmetry or possible overlapping of more than two bands similar to that of $\alpha(Fe)_2\beta(Co)_2$.[15] For the cross-linked hybrid with two α(Fe) subunits, the band intensity increased without a change of the band shape. Therefore, the abnormal band shape cannot be attributed to the difference between two α subunits. It is likely that the strain imposed on the proximal histidine of the α subunit is not homogeneous in all Hb molecules present but has some statistical distribution, which results in inhomogeneous broadening of the ν_{Fe-His} band.

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On the other hand, the ν_{Fe-His} band of the tetramer with a single β(Fe) subunit is fairly symmetric (Figure 2A), and it is intensified without a change of the band shape for the double β(Fe) species (Figure 3A). This may suggest homogeneity of the β subunits which have much less strain in the Fe–His bond. However, the ^{1}H NMR signal of proximal histidine of α(Co) is much sharper than that of β(Co) for both $[\alpha(Fe)\beta(Co)]_A[\alpha(Co)\beta(Co)]_CXL$ and $[\alpha(Co)\beta(Fe)]_A[\alpha(Co)\beta(Co)]_CXL$.[19] A reason for an apparent discrepancy between the Raman, ESR, and NMR results is not clear, but it might arise from difference between the bonding character of the Fe–His and Co–His bonds, which is noticed in the fact that the Fe–His stretching RR band can be resonance-enhanced but the Co–His stretching RR band has never been identified for deoxyCoHb.

The ν_{Fe-His} frequency of the photodissociated transient COFe subunit is considered to reflect qualitatively the nature of the Fe–His bond in the CO-bound form, although, in the present time resolution of 50 μs, the protein structure might be somewhat relaxed toward the equilibrium deoxyHb.[30] Here, the RR spectrum in Figure 1B can serve as a standard of the transient form in the present time resolution. The behavior of the ν_{Fe-His} band during the oxygenation process was investigated by Spiro and co-workers[34] who noted that the band of deoxyHb at 215 cm^{-1} shifted to 219 cm^{-1} at an oxygenation level of 75% for Hb A. This frequency is still lower than that of the photodissociated transient deoxyHb A shown in Figure 1B. For the mono-ligated species shown in Figure 4B, the transient ν_{Fe-His} band of the β(COFe) subunit is shifted appreciably to higher frequencies than that of the deoxy species, but definitely does not reach the frequency of spectrum C of Figure 4. This means that the Fe–His bond of the β(COFe) subunit in the $\alpha(Co)_2\beta(COFe)\beta(Co)$ adopts a state different from those of deoxyHb and fully ligated Hb. In contrast, the transient ν_{Fe-His} band of the α(COFe) subunit remained practically unshifted in spectrum B′, suggesting that the Fe–His bond of the α(COFe) subunit in $\alpha(COFe)\alpha(Co)\beta(Co)_2$ resembles that of deoxyHb. The NMR spectra of the mono-ligated species[19] revealed that binding of CO to the α(Fe) subunit does not change the proximal histidine signals of the remaining three subunits as well as the T-state marker of the protein at 10.8 ppm but reduces the intensity of the T-state marker at 14.1 ppm to half. On the other hand, the binding of CO to a single β(Fe) subunit resulted in coalescence of the two proximal histidine signals of the remaining α(Co) and β(Co) subunits to a single peak, disappearance of the T-state marker around 14 ppm, and intensity reduction of the other T-state marker at 10.8 ppm. Although the NMR signals of proximal His reflect a state of nonligated subunits, these results are consistent with the present results in the sense that the binding of CO to the β subunit causes a larger structural change of the tetramer than its binding to the α subunit and, as a result, the ν_{Fe-His} mode of the CO-bound heme itself undergoes a larger change toward reduction of the strain, giving rise to the ν_{Fe-His} frequency of neither typical T nor R type. This implies the presence of another quaternary structure in the intermediate oxygenation states besides the two typical T and R quaternary structures. When the three Co subunits are oxygenated, the photodissociated transient COFe subunit is considered to adopt the R structure and, accordingly, the difference between the α and β subunits is dissolved.

The results shown in Figure 4, spectra B and B′, indicate that, when two CO molecules are bound either to the α or β subunits, the tetramer adopts the R structure and therefore the Fe–His bonds of the photodissociated transient CoFe subunits are not altered by binding of oxygen to the remaining two Co subunits. This is consistent with the NMR study which pointed out that the doubly ligated tetramer had no signal of the T-state marker of the protein moiety.[17] Consequently, the Raman and NMR studies suggest that a quaternary structure changes upon binding of one or two ligands. This is consistent with the change of the oxygen equilibrium properties of mono-CO asymmetric Fe–Co hybrids at K_3 stage.[20] In the case of Hb A, presumably, a similar structure change occurs when three ligands are bound. Although there might be more than two structures during the oxygenation process, the present results would not favor a simple sequential model which assumes the presence of five distinct structures.

The Fe–CO Stretching Mode. Yu and co-workers[26a,b] systematically studied the correlation between the ν_{Fe-CO} frequencies and steric hindrance to the binding site of CO and concluded that the increase of the steric hindrance raises the ν_{Fe-CO} frequency; only when the Fe–CO linkage is distorted from a linear arrangement, due to the steric hindrance, does the FeCO bending mode gains RR intensity. According to their results, the ν_{Fe-CO} frequency is expected at 495 cm^{-1} for unhindered conditions and it shifts to 514 cm^{-1} upon distortion, which is close to the ν_{Fe-CO} frequency of CO–myoglobin (COMb).[35] The X-ray crystallographic analysis of deoxyHb[36] revealed that Val-E11 of the β subunit occupies the ligand binding position. Thus, it was deduced that there would be steric hindrance for the CO molecule to bind to the β subunit. The results shown in Figures 6 and 7 indicate that the ν_{Fe-CO} frequency is slightly lower for the β than α subunits. This is opposite to the prediction by Yu et al.[26a] for the presence of the steric hindrance in the β subunit. Furthermore, the frequency difference of the ν_{Fe-CO} mode between COHb (507 cm^{-1}) and COMb (512 cm^{-1})[35] is much larger than the difference between the α and β subunits. Therefore, it is plausible that the tertiary structure of the β subunit is more flexible than the α subunit, and Val-E11β is easily moved away upon binding of a ligand to the β subunit so that the resulting structure could not impose a significant strain on the bound CO molecule. We emphasize that this structure change includes cleavage of the hydrogen bond between Tyr-42α and Asp-99β, and thus induces intensity reduction or disappearance of the T-marker NMR signal at 14 ppm. This interpretation is consistent with the recent results that the ν_{Fe-CO} frequency remained unchanged when Val-E11β was replaced by Ile, Ala, Leu, and Met by using a technique of the protein engineering.[37]

The OO Stretching Mode. A hydrogen bond between the bound oxygen and distal histidine would be expected to stabilize the oxy form of the protein and could contribute to the regulation of the oxygen affinity. Its presence has been proposed by Pauling,[38] and the supporting evidence have been accumulated from a series of ESR and functional studies on various Co-substituted Hb and Mb.[39,40a–c] This was established by neutron diffraction study for crystals of oxyMb.[41] For oxyHb Schaanan[42] pointed out from an X-ray crystallographic analysis that distal His of the α subunit forms a hydrogen bond with the bound oxygen, but the corresponding hydrogen bond of the β subunit would be much weaker because of the increased distance. Since the OO stretching vibration of oxyCoHb exhibits a frequency shift of 2–4 cm^{-1} upon H_2O–D_2O exchange,[24] the ν_{OO} frequency is expected to depend on the strength of hydrogen bond. The results shown in Figure 8 indicate that the ν_{OO} frequencies of the α and β subunits are practically the same. The apparent discrepancy between the present results and the X-ray results might be caused by the difference between the Co and Fe hemes, for which the out-of-plane displacement of the metal ions might differ between the two

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hemes. Alternatively, the difference in the location of distal His between the α and β subunits is not so effective as yielding appreciable difference in the bond energy of the bound oxygen. The invariance of the ν_{OO} frequency between the α and β subunits is also seen for the tetramer containing photodissociated transient COFe subunits as shown in Figure 9. Since we failed to obtain the ν_{OO} frequency for the tetramer having the equilibrium deoxyFe subunits, the role of this hydrogen bond in a quaternary structure change is not clear at the present stage. The ν_{OO} mode for oxy-Co-Ni hybrid, in which the Ni subunit mimics the deoxyFe subunit,[43] may provide important information about a change of the hydrogen bond upon a change of quaternary structure.

Acknowledgment. This work has been in part supported by Research Grant HL 14508 from the National Heart, Lung, and Blood Institute and Grant-in-Aid for Cooperative Research (60304099) from the Ministry of Education, Science and Culture.

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Dynamic Exchange and Rotational Behavior of 2-Methylallyllithium: ^1H, ^{13}C, and ^6Li NMR

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Abstract: Proton NMR of 2-methylallyllithium-^6Li (**1**) in diethyl-d_{10} ether at 150 K indicates the existence of two species, differing in aggregation, undergoing interconversion and internal rotation slowly relative to the NMR time scale. NOE experiments, $^1H\{^1H\}$ and $^6Li\{^1H\}$, establish shifts for methylene protons syn and anti to methyl and show lithium to be closest to the anti protons. NMR line-shape analysis of **1**, in diethyl-d_{10} ether above 150 K, yields $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for rotation of 9.6 kcal and −7.3 eu, whereas for interspecie exchange these values are 7.3 kcal and −3.4 eu. In the presence, separately, of THF, TMEDA, and pentamethyldiethylenetriamine (PMDTA) the predominant form of **1** is the less associated species. It is proposed that the line broadening seen previously in low-temperature (200 K) NMR spectra of allyllithiums resulted from exchange phenomena rather than field inhomogeneities from viscosity and precipitation effects.

Allyllithium is the simplest potentially conjugated of all organometallic compounds.[1,2] It is therefore surprising that despite extensive studies NMR,[3] crystallographic,[4] and theoretical[5,6] there are still fundamental unanswered questions. These include its structure(s) in solution, especially the site(s) of lithium in relation to the allyl moiety. Most organolithium compounds exist as rapidly interconverting mixtures of different species whose distribution varies with temperature and concentration.[6] Such data are not currently available for allyllithium. In all likelihood the rates of specie interconversion have been too fast to handle with NMR methods, at the lowest temperatures used so far. Barriers to rotation of allyllithiums have been measured with NMR methods.[3a,7-9] It is not clear whether these apply to single species or are averages over some distribution of species.

This paper reports new insights into the structure and dynamic behavior of 2-methylallyllithium which we have studied using a variety of NMR methods, starting at very low temperatures.

Results and Discussion

Cleavage of tetrakis(2-methylallyl)tin by butyllithium in diethyl ether yielded clean samples of 2-methylallyllithium; see Experimental Section.

Proton NMR, 500 MHz, of **1** ca. 0.05 M in diethyl-d_{10} ether at room temperature consists of one sharp peak each for CH_2 and CH_3 at δ 2.15 and 1.7, respectively. There is no evidence of spin coupling between CH_2 and CH_3. With decreasing temperature the CH_2 resonance broadens and splits out into two δ peaks, by 205 K at δ 2.32 and 1.98, reminiscent of results attributed to slow rotation about the C_1–C_2 (C_2–C_3) bonds of other allyllithiums.[3a,7-9] However, below 205 K the δ 2.32 peak broadens, disappears into the base line, and develops by 155 K into two lines centered at δ 2.15 and 2.84, respectively; see Figure 1. Throughout this temperature range (205 to 155 K) and in other samples as low as 135 K, the δ 1.98 peak remains sharp and its shape does not change, as is also the case for the methyl resonance which is always a single line. Cooling the ether solution of **1** below 155 K brings about some line broadening but no significant changes. However, when **1** is diluted in diethyl ether (see Figure 2), the area ratio of the resonance at δ 2.15 to that at δ 2.84 increases markedly, implying the existence of at least two species, differing in aggregation. Furthermore, the summed area at δ 2.15 and 2.84 always equals that at δ 1.98. Then it appears that each of two species gives rise to two methylene proton resonances: at δ 2.84 and 1.98 for the prevailing species at higher concentrations of **1** and δ 2.15 and 1.98 for the less associated species. Note that the singlet at δ 1.98 represents methylene protons from *two* species.

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