Nonequivalence in the Electronic Structure of the Prosthetic Groups between Two $\alpha$-Subunits within Deoxycobalthemoglobin as Determined by Single-crystal EPR Spectroscopy*

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An artificial hybrid hemoglobin, $\alpha$(Co)$_2$$\beta$$(Fe)_2$, the $\alpha$- and $\beta$-subunits of which contain cobaltous and ferrous protoporphyrins IX, respectively, and its complementary hybrid, $\alpha$$(Fe)_2$$\beta$(Co)$_2$ were prepared from human hemoglobin, crystallized in the deoxy state, and examined by electron paramagnetic resonance (EPR) spectroscopy. The orientations of the porphyrin normals in these deoxy Fe-Co hybrid hemoglobins in terms of the $g_\parallel$ signals, were closely coincident with those of the heme normals of deoxyhemoglobin determined by x-ray crystallography.

Two sets of axially symmetric EPR signals were found in the $\alpha$(Co)-subunits, whereas only one set was observed in the $\beta$(Co)-subunits. Nonequivalence in the electronic structures of the prosthetic groups between the two $\alpha$(Co)-subunits, designated $\alpha_1$ and $\alpha_2$, within deoxy-$\alpha$(Co)$_2$$\beta$(Fe)$_2$ hybrid hemoglobin was correlated to these two distinct EPR signals. The interaction between the $\gamma$-nitrogen of the imidazole ring of the proximal histidine and the cobaltous ion in deoxy-$\alpha$(Co)$_2$-subunit is different from that in the deoxy-$\alpha$(Fe)$_2$-subunit. The absence of a strict molecular dyad axis in the deoxy-$\alpha$(Co)$_2$$\beta$(Fe)$_2$ hybrid hemoglobin suggests that the affinity state of the $\alpha$(Co)-subunits may be partially switched to the R-state having a higher affinity for oxygen.

Upon partial ligation of carbon monoxide to the $\beta$(Fe)-subunits, the line width of the $g_\parallel$ and $g_\perp$ signals of the $\alpha$(Co)-subunit was found to become somewhat narrower without disruption of the crystal structure. This suggests that there may be very close contacts between the $\alpha$- and $\beta$-subunits of different hemoglobin molecules which appear to be responsible for stabilizing the deoxy crystal structure after partial ligation in the crystalline state.

The cooperative oxygen binding in hemoglobin has been interpreted to be derived from a reversible transition between two alternative quaternary structures (1, 2). X-ray crystallographic studies of Hb (2) had revealed two distinct quaternary structures, designated the T- and R-states having low and high oxygen affinities, respectively. The information of the electronic states and the functional properties of the prosthetic groups in these two different quaternary states and/or in the intermediate states of oxygenation are vital in our understanding of the detailed molecular mechanism of the cooperativity in Hb.

The chemical substitution of the ferrous protoporphyrin prosthetic groups with cobaltous protoporphyrins has allowed detailed investigations of the mode of interactions among deoxygen, the prosthetic groups, and the apoprotein moiety in Hb, since the cobaltous-substituted Hb exhibits electron paramagnetic resonance (EPR) spectra in both deoxy and oxy states, and it substantially retains the cooperativity in the oxygen binding and allosteric properties (8-5).

Previously, Ikeda-Satto et al. (6) investigated EPR properties of Fe-Co hybrid Hbs. They noted that the EPR spectrum of deoxy-$\beta$(Co)-subunits within deoxy-$\alpha$(Fe)$_2$$\beta$(Co)$_2$ was identical with that of the isolated deoxy-$\beta$(Co) chains, whereas the EPR spectrum of deoxy-$\alpha$(Co)-subunits within deoxy-$\alpha$(Co)$_2$$\beta$(Fe)$_2$ was different from that of the isolated deoxy-$\alpha$(Co)-chains and strongly sensitive to pH and the presence of allosteric effectors. Tsubaki and Nagai (7) and, more recently, Inubushi and Yonetani (8) reported EPR studies on Co-Hb and Fe-Co hybrid Hbs in order to determine the relationship between the electronic state of the prosthetic group and the quaternary states for these Co$^{2+}$-substituted Hbs. These investigators reported that the EPR spectra of deoxy-$\alpha$(Co)$_2$$\beta$(Fe)$_2$ can be interpreted to have derived from a mixture of two distinctive paramagnetic species, where one has a broad signal and another a narrow signal. The oxygen equilibrium data of this hybrid (7) indicate that the former may be assigned to the low affinity T-state and the latter to the high affinity R-state. The relative ratio of the intensities of these two EPR signals was found to be related to the ratio of the corresponding two quaternary structures present. However, the powder EPR study cannot distinguish whether these two EPR signals are derived from two different $\alpha$(Co)-subunits between two Hb molecules having different quaternary structures or from the different electronic states between two $\alpha$(Co)-subunits within one $\alpha$(Co)$_2$$\beta$(Fe)$_2$ hybrid Hb molecule. In this paper, we have analyzed single-crystal EPR spectra of deoxy-CoHb and deoxy Fe-Co hybrid Hbs in order to investigate the relationship between the electronic structure of the prosthetic group and the structure of the globin moiety. The EPR data on deoxy-Co$^{2+}$-substituted Hb crystals are presented.

**EXPERIMENTAL PROCEDURES**

HbA was prepared from human blood by the method of Drakkin (9). CoHb and Fe-Co hybrid Hbs were prepared according to the methods reported previously (4, 6, 10). Deoxygenation of the CoHb and Co-Fe hybrid samples was achieved by anaerobic addition of solid
sodium dithionite. Crystals of deoxy-CoHb and deoxy Co-Fe hybrid Hbs were grown from an ammonium sulfate/phosphate solution, as described for native deoxy-HbA by Perutz (2). The partially ligated Fe-Co hybrid crystals were prepared by gently bubbling carbon monoxide gas through the mother liquor containing fully deoxy crystals for several minutes.

EPR measurements were carried out at 3.73 GHz (S-band) and 9.23 GHz (X-band) with EPR components from Micro-Now Instrument Co. (Skokie, IL) and Varian Associates (Palo Alto, CA), respectively. An immersion Dewar flask was used for X-band measurements at 150°C and a Varian variable-temperature Dewar was used for S-band measurements above 77 K. A home-built, two-circle Teflon goniometer with an angular precision of +5° was used for rotation of single-crystal specimens in EPR Dewar flasks.

RESULTS

A crystal of fully deoxy-CoHb has a space group of P2₁, and is isomorphous with that of deoxy-HbA (12). Since its unit cell contains two Hb molecules, eight sets of EPR signals corresponding to eight Co(II) ions in the unit cell can be expected in an arbitrary crystalline plane. Because of its spectral complexity, however, it was technically difficult to accurately determine the porphyrin normals (the z-axes) in the deoxy-CoHb crystal. On the other hand, in crystals of deoxy Fe-Co hybrid Hbs, only four sets of EPR signals were expected, making the EPR spectral analysis substantially simpler. Fig. 1 illustrates the X-band EPR spectra of single crystals of fully deoxy-α(Fe)β(Fe)₂ and its complementary form, α(Fe)₀β(Fe)₂, and of deoxy-CoHb. Recorded with an applied magnetic field perpendicular to each one of the porphyrin planes of the four Co²⁺-containing subunits in the unit cell. A well-resolved octuplet ⁵⁹Co-hyperfine splitting with a triplê ¹⁴N-superhyperfine splitting was observed. A maximal value of the Co²⁺-hyperfine coupling constant (A⁵⁹Co) and a minimal g-value (g₂) were observed along the porphyrin normal. It was reasonable to consider that the g-tensor and the Co²⁺-hyperfine coupling tensor shared the same principal axes, as was the case for CoMb (13, 14). Less-resolved EPR signals of the other Co²⁺-containing subunits in the unit cell were observed in the low-field side under these conditions, as shown in Fig. 1. The line shape of the EPR signal of the deoxy-α(Fe)β(Fe)₂ subunit within α(Fe)₀β(Fe)₂ was different from that of the deoxy-β(Fe)₂ subunit within α(Fe)₀β(Fe)₂. The difference was particularly noticeable in the high field extreme. The line width of the g₁ signal of the deoxy-α(Fe)₀β(Fe)₂ crystal was larger than that of the deoxy-α(Fe)₀β(Fe)₂ crystal as expected from powder EPR spectra of these Fe-Co hybrid Hbs (6). The g₁ or g₂ signal of one of the two β(Fe)₂ subunits within the deoxy-α(Fe)₀β(Fe)₂ tetramer, designated β₁(Fe), was indistinguishable from that of another β(Fe)₂ subunit, designated β₂(Fe). On the other hand, the g₁ or g₃ signal of the α₁(Fe)β(Fe)₂ subunit was found to be distinctly different from that of the α₁(Fe)β(Fe)₂ subunit where α₁(Fe) and α₁(Fe) represented two Co²⁺-substituted subunits within the deoxy-α(Fe)₀β(Fe)₂ tetramer.

The directions of the porphyrin normal (the z-axes) of these Co²⁺-substituted subunits within Fe-Co hybrid tetramer crystals are indicated in the stereographic projections in Fig. 1. The g₁ or g₃ values, the hyperfine coupling constants (A⁵⁹Co and A¹⁴N) and the orientations of the z-axes relative to the crystallographic axes are listed in Table I. The porphyrin normals (the z-axes) for α₁(Fe), α₁(Fe), β₁(Fe), and β₁(Fe) subunits lie nearly in the crystallographic ab plane and make angles of 20°, 45°, 10°, and 35°, with the crystallographic a axis, respectively. From the orientation of these z-axes, the molecular dyad axis, if present, was determined to make angles of 79°, 11°, and 94° with crystallographic a-, b-, and c*-axes, respectively. The orientations of the porphyrin normals and the molecular dyad axis for a fully deoxy-CoHb crystal were reported to be identical with those of native deoxy-HbA (15, 16). The coordinates were provided by the Data Bank of Osaka University.

As the z-axes and the molecular dyad axis of fully deoxy Fe-Co hybrid Hb crystals lie nearly in the ab plane and, in addition, the 2-fold symmetry axis of these crystals lies along the crystallographic b-axis, a set of four EPR signals were expected to be equivalent, when the magnetic field was applied in the direction of the crystallographic c*-axis. This signal would represent one of the EPR signals in the porphyrin plane. If the α₁(Fe) and α₁(Fe)-subunits or β₁(Fe)- and β₁(Fe)-subunits were not equivalent within the Fe-Co hybrid crystals, two types of EPR signals would be observed in the vicinity of the c*-axis. In fully deoxy-α(Fe)₀β(Fe)₂, the EPR signal (g₁) corresponding to the β(Fe)₂ subunits was observed in the vicinity of the c*-axis as shown in Fig. 24, indicating the equivalence of two β(Fe)₂ subunits. In contrast, X-band EPR spectra of fully deoxy-α(Fe)₀β(Fe)₂ crystals showed that

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** EPR spectra of single crystals of the fully deoxy-α(Fe)₀β(Fe)₂ hybrid (upper two spectra) and fully deoxy-α(Fe)₀β(Fe)₂ hybrid (lower two spectra) in the g₁ region at 77 K. Each Co-porphyrin normal (the z-axis) is shown on the stereographic diagrams. These z-axes belong to another Hb molecule and can be shown by a symmetrical operation of a single crystal (the b-axis crystallographic 2-fold symmetrical axis) and, thus, is not shown in these diagrams. The direction of the molecular dyad axis is also indicated by a symbol ( ).

<table>
<thead>
<tr>
<th>Fe-Co hybrid Hb</th>
<th>Principal values</th>
<th>Angle to c*</th>
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<tbody>
<tr>
<td>α₁(Fe)β(Fe)₂</td>
<td>g₁ A¹⁴N A⁵⁹Co</td>
<td>a b c*</td>
</tr>
<tr>
<td>2.040</td>
<td>7.7 1.7</td>
<td>20° 70° 90°</td>
</tr>
<tr>
<td>2.038</td>
<td>8.0 1.7</td>
<td>-45 45 90</td>
</tr>
<tr>
<td>α(Fe)₀β(Fe)₂</td>
<td>β₁ A¹⁴N A⁵⁹Co</td>
<td>a b c*</td>
</tr>
<tr>
<td>2.037</td>
<td>7.8 1.7</td>
<td>-10 -80 88</td>
</tr>
<tr>
<td>2.035</td>
<td>7.8 1.7</td>
<td>-35 56 80</td>
</tr>
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tensors because of the complex tensor, as well as the single-crystal study has been carried out in order to analyze the A? at X- and S-bands (B) in the vicinity of the crystallographic c*-axis. X-band spectra were measured at 77 K, and the S-band spectrum was measured at -150 °C.

The g signal in the vicinity of the c*-axis consisted of a mixture of two EPR species,1 one abnormally broad signal and the other narrower one with partially resolved 69Co-hyperfine splittings, as shown in Fig. 2B. In S-band EPR spectra, two sets of the 69Co-hyperfine couplings were more clearly observed in the vicinity of the c*-axis, as shown in Fig. 2B. These results clearly indicated that the electronic states of the Co? ions in the two a(Co)-subunits, a1(Co) and a2(Co), within a fully deoxy-α(Co)?(Fe)? hybrid crystal were not identical in the directions both along the porphyrin normal and in the porphyrin plane. As reported previously (6), the anomalously broad powder EPR signal of fully deoxy-α(Co)?(Fe)? was converted to a normal narrower EPR signal upon ligation of carbon monoxide to the β(Fe)2-subunits. Single-crystal EPR spectra of fully deoxy- and partially CO-ligated α(Co)2β(Fe)2 hybrids measured with an applied magnetic field parallel to one of the z-axes of the α(Co)-subunits in the unit cell. As the z-axes corresponding to two α(Co)-subunits made an angle of 90° with each other in the ab plane, the g signal of one of the α(Co)-subunits overlapped with the g signal of the other α(Co)-subunit in this direction, as shown in Fig. 3A. Although the other two EPR signals of the α(Co)-subunits also overlapped with these g1 (g2) and g signals of the α(Co)-subunits, the signal intensity of the latter was predominant in this particular direction. Upon partial ligation of carbon monoxide to the β(Fe)2-subunits in the same crystal, line widths of both the g1 (g2) and g signals, especially the latter, of the α(Co)-subunits became narrower, as shown in Fig. 2B. These results were in good agreement with those of powder EPR experiments (6). Full ligation of carbon monoxide to the β(Fe)2-subunits induced the disappearance of the single-crystal EPR pattern in the α(Co)2β(Fe)2 hybrid, indicating the disruption of the crystal lattice structure.

1 Although g signals corresponding to α(Co)- and α(Co)-subunits were partially resolved by the hyperfine coupling due to the 69Co nucleus with a spacing of 1.5 and 2.6 milliTesla, respectively, the principal A? values are still unknown. Our recent EPR study on deoxycyoglobin-substituted myoglobin crystal has revealed that the A? hyperfine coupling is somewhat anisotropic in the porphyrin plane (14). Therefore, we should consider that deoxy-CoHb may also exhibit an anisotropic A? hyperfine coupling in the porphyrin plane. A full single-crystal study has been carried out in order to analyze the A? tensor, as well as the g tensor. However, we failed to analyze these tensors because of the complex EPR signals overlapping with signals from four Co? substitutes in the unit cell. Further analysis is under way by S-band EPR spectroscopy for both powder and single-crystal specimens of Fe-Co hybrid Hbs.

DISCUSSION

Orientations of the Prosthetic Groups in Deoxy-CoHb Crystals—It has been previously assumed that the 69Co-hyperfine splitting tensor and the g tensor of deoxy-CoHb are axially symmetric and share the same principal axes (1, 9, 17). The axial A? or A? (or A? or A?) values are readily estimated from powder EPR spectra which exhibit well-resolved octuplet 69Co-hyperfine splitting with a triplet 14N-superhyperfine splitting due to proximal histidine. The identification of the unique z-axis in the tetragonal ligand fields is conveniently aided by an abrupt onset of a well-resolved 69Co-hyperfine splitting in the EPR spectrum of a deoxy-CoHb crystal. This axis is assumed to be coincident with the porphyrin normal and has been found to be virtually identical with the g (or g) direction.

In the present single-crystal EPR study, the orientations of the porphyrin normals corresponding to the α(Co)- and β(Fe)-subunits were separately determined using deoxy-α(Co)2β(Fe)? and deoxy-α(Co)2β(Fe)2 hybrid crystals, respectively. Since the Fe?2-containing subunits were EPR-silent, the observed EPR signals reflected only the electronic state of the Co(II)-porphyrins in the Fe-Co hybrid Hbs. Moreover, powder EPR spectra of deoxy-CoHb tetramers were found to be an arithmetic sum of those of fully deoxy-α(Co)2β(Fe)? and fully deoxy-α(Co)2β(Fe)? hybrid crystals, in agreement with a previous observation (6). Therefore, it was assumed that this relation would be maintained in single-crystal EPR data. The orientations of these porphyrin normals (the z-axes) corresponding to the Co(II)-containing subunits in single crystals are listed in Table 1. These orientations are in close agreement with those of the heme normals of deoxy-HbA which were determined by an x-ray crystallographic analysis (16). Thus, it is reasonable to consider that the (x, y, z)-axis system is fixed in the framework of the porphyrins.

Furthermore, the orientation of the molecular dyad axis for fully deoxy-CoHb, which was calculated from the orientations of these four z-axes, exactly coincides with that of native deoxy-HbA (15). A comparison of x-ray crystallographic structures of human deoxy-CoHb and deoxy-HbA by Fermi et al. (12) shows that the structure of the globin moiety and the orientation of the prosthetic groups are not significantly changed upon the substitution of the heme groups with Co(II)-porphyrins. Our present results on the Fe-Co hybrid Hbs also agree well with those of the x-ray crystallographic study. On the other hand, Dickinson and Chien (17) reported from a single-crystal EPR study that the orientations of the porphyrin normals in horse deoxy-CoHb were significantly different from those in horse MetHb. It should be pointed out
that, in their experiments, crystals of horse deoxy-CoHb were prepared by reduction of crystals of horse Met-CoHb or Co(III)Hb with dithionite. It is likely that certain changes in the globin conformation may take place upon reduction of Met-CoHb crystals to deoxy-CoHb crystals with no apparent fracture of the lattice structure.

Nonequivalence in the Electronic Structure between Two α(Co)-Subunits within Tetrameric Deoxy-CoHb—Our present EPR study clearly demonstrates that the electronic structures of the Co(II) ions corresponding to two deoxy-α(Co)-subunits, α₁(α) and α₁(α), are different from each other within a fully deoxy-(Co)₃β(Fe)₂ hybrid molecule, although the globin structures as well as the porphyrin normals of these two α(Co)-subunits are exactly 2-fold symmetrical with respect to the molecular dyad axis. This unexpected result was not due to the crystalline disorder of a sample crystal, as we had been able to reproduce the same result with every one of the crystal samples which were prepared from different batches of the Fe-Co hybrid Hbs. The difference in the electronic state between the β₁(αC)- and β₁(αC)-subunits, if present, was found to be insignificant, as shown in Fig. 1. This result is consistent with a power EPR study (6, 7) showing that powder EPR spectra of deoxy-β(Co)-subunits were not significantly affected by structural changes in a tetrameric deoxy-CoHb.

It has been previously assumed that the two α-subunits and/or the two β-subunits are equivalent to each other in a tetrameric Hb molecule. X-ray crystallographic analyses of proteins apply a technique of symmetry-averaging about the molecular dyad axis in order to obtain higher resolution. The direction of the molecular dyad axis, however, does not coincide with that of the crystallographic 2-fold symmetry axis in deoxy-HbA and deoxy-CoHb crystals (12, 15). Fig. 4 illustrates the two-dimensional representation of the contact between neighboring deoxy-Hb molecules in the asymmetric unit cell. The contact between the α₁-subunit of one Hb molecule and the β₁-subunit of the other Hb molecule is different from the contact between the α₁-subunit of the former and the β₁-subunit of the latter. Therefore, the α₁ and α₂-subunits, as well as the β₁ and β₂-subunits, are not coincident with each other at least in the asymmetric unit cell. The identical circumstance is expected in the unit cells of deoxy-CoHb and deoxy Fe-Co hybrid Hbs. Since our present single-crystal EPR data indicate the absence of a strict molecular dyad axis in the deoxy-α₁(α)β(Fe)₂ hybrid crystal, it may be reasonable to assume that the molecular structure of the tetrameric deoxy-α₁(α)β(Fe)₂ hybrid Hb has an asymmetric structure in the crystal, where one may state that nonequivalent electronic states between the two α(Co)-subunits within the deoxy-α_1(α)β(Fe)₂ hybrid might be derived from the unique intermolecular geometry in the crystal.

Crystals of horse MetHb and horse CoHb are monoclinic with space group C₂, containing only two molecules per unit cell. The direction of the molecular dyad axis coincides with that of the crystallographic 2-fold symmetry axis in these crystals (17, 18). Therefore, if there was no such nonequivalence between two α(Co)-subunits, as well as between two β(Co)-subunits within the crystal of horse CoHb, the inequivalence observed between the two α(Co)-subunits in single crystals of human deoxy-α₁(α)β(Fe)₂ could be due to the crystal packing effect of the sort indicated above. Such an inequivalence was not detected in the earlier single-crystal EPR work (17). In addition, the effect of the partial ligation of carbon monoxide on the EPR spectrum of a single crystal of deoxy-α₁(α)β(Fe)₂ hybrid requires some comment. Upon full ligation of carbon monoxide to the β(Fe)-subunits, the anomalously broad power EPR signal of fully deoxy-α₁(α)β(Fe)₂ hybrid changes to a more normal narrow EPR signal (6). This indicates that the quaternary structure of α₁(α)β(Fe)₂ hybrid switches from the T-state to the R-state upon ligation of carbon monoxide to the β(Fe)-subunits (6). Present single-crystal EPR data demonstrate that, upon partial ligation of carbon monoxide to the β(Fe)-subunits within the same hybrid in the crystal, the line width of the g₁ and g₂ signals corresponding to the α₁(α)-subunit becomes somewhat narrower without disruption of the T-state crystal structure. The change in the g signal is particularly noticeable. The change in the quaternary structure of α₁(α)β(Fe)₂ hybrid and/or the change in the localized strain at the prosthetic group imposed by globin within a given quaternary structure may occur upon partial ligation of carbon monoxide to at most one of the β(Fe)-subunits without disruption of the crystal lattice structure.

Thus, the nonequivalence in the electronic structure between the two α(Co)-subunits within the tetrameric deoxy-α₁(α)β(Fe)₂ hybrid might not be derived from the unique intermolecular geometry in the crystal, but might be originated from the intrinsic quaternary structure of the deoxy α₁(α)β(Fe)₂ hybrid molecule.

The powder EPR data on deoxy-α₁(α)β(Fe)₂ hybrid, where there would not be such a specific intermolecular interaction as would be possible in the crystalline state, indicated that there exists a mixture of two distinct paramagnetic species (8). It was not possible to determine whether these two species were derived from the two α(Co)-subunits within a single tetrameric hybrid or there exist two populations of different deoxy-α₁(α)β(Fe)₂ hybrid molecules having different quaternary structures in equilibrium solutions. A comparison of the present single-crystal EPR results on deoxy-α₁(α)β(Fe)₂ with the corresponding powder EPR data (8) provides a reasonable structural interpretation. The narrower EPR signal with a well-resolved triplet ¹⁴N-superhyperfine splitting of the α₁(α)-subunit represents an unhindered ligation of the proximal histidine to the Co(II) ion, whereas the broader EPR signal with a less well-resolved ¹⁴N-superhyperfine splitting of the α₁(α)-subunit may be interpreted as a constrained or weak ligation of the proximal histidine to the Co(II) ion, leading to a lower ligand affinity. Both of these α₁(α) coordination states exist within the same Fe-Co hybrid molecule.

Furthermore, the recent x-ray crystallographic study on the
partially oxygenated T-state Hb crystal, $\alpha(\text{Fe}-\text{O}_2)\beta(\text{Fe})_2$ (19), indicates that the positions and contacts of the oxygen molecules in the two $\alpha$(Fe)-prosthetic groups are different, although the Hb quaternary structure has not been altered. Moreover, the $N_c$-iron distance was reported to be somewhat lengthened in one of the $\alpha$(Fe)-subunits (19). Our single-crystal EPR data on deoxy-$\alpha(\text{Co})\beta(\text{Fe})_2$ hybrid indicates that the electronic states of Co(II)-protoporphyrins in the two $\alpha$(Co)-subunits are different, although the crystal structure of this hybrid seems to be stabilized as a deoxy T-state. In this particular crystal, the $\alpha(\text{Co})\beta(\text{Fe})_2$ hybrid appears to be in an intermediate state between the unligated T- and ligated R-states. The affinity state of one of the two $\alpha$(Co)-subunits, the $\alpha_1$(Co)-subunit, may be partially switched to the higher oxygen affinity state or the R-state. Our results also suggest that there may be very close contacts between the $\alpha$- and $\beta$-subunits of different Hb molecules in a crystal as illustrated in Fig. 4, which appears to be responsible for the stabilization of the deoxy structure after partial ligation in the crystalline state. Further studies of precise x-ray crystallography on fully deoxy-CoHb and Fe-Co hybrid Hbs may be required to unequivocally determine the detailed mechanism of such stabilization.

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REFERENCES