The Porphyrin-Iron Hybrid Hemoglobins

ABSENCE OF THE Fe-His BONDS IN ONE TYPE OF SUBUNITS FAVORS A DEOXY-LIKE STRUCTURE WITH LOW OXYGEN AFFINITY*

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Protoporphyrin-protoheme hybrid hemoglobins (Hb), in which the protophemes (Fe) in either the α- or β-subunits were substituted with protoporphyrins IX (PP) α(PP)β(Fe₂) and α(Fe)β(PP₂) have been prepared. The structural and functional properties of these hybrid Hbs were investigated by measuring oxygen equilibrium curves and proton nuclear magnetic resonance spectra. The equilibrium constants of the first ligand, K₁, observed for α(PP)β(Fe₂) were much smaller than K₁ values of HbA. The effects of pH and inositol hexaphosphate on K₁ were substantially diminished. On the other hand, K₁ values of α(Fe)β(PP₂) were similar to those of HbA, including the pH and inositol hexaphosphate effects. The deoxy forms of α(PP)β(Fe₂) and α(Fe)β(PP₂) showed exchangeable proton resonances at 11 and 14 parts/million arising from the hydrogen bonds at the α₁β₂ contact in a deoxy-like structure. In the liganded form, these signals were dependent upon solution conditions. As K₁ became larger, the reduction in the intensity of these signals was observed for both liganded forms. The resonance position of E₁1 Val originating from the β subunits of α(PP)β(Fe-CO)₂ also varied in accordance with K₁. We compare properties of PP-Fe hybrids with those of Co-Fe and Ni-Fe hybrids and conclude that the first oxygen binding to the β heme may be linked to the metal-proximal His interaction in the α subunits. However, the first oxygen binding to the α heme is linked minimally to the metal-proximal His interaction in the β subunits but may be correlated instead to the position of E₁1 Val relative to the porphyrin plane in the β subunits.

Hemoglobin cooperativity originates from the conformational change associated with ligand binding. Based on differences in heme stereochemistry between oxy- and deoxy quaternary structures, Perutz (1970) assigned a critical role to the behavior of the Fe-His(F8) bond which is the only covalent

 linkage between the heme and the globin moieties in Hb. A specific role in modulating the ligand affinity and in triggering the allosteric transition has been attributed to this linkage. In this regard, the original framework of Perutz’s stereochemical theory does not particularly distinguish the role of the Fe-His(F8) bonds between α and β subunits. However, the Fe-His(F8) bonds in the α and β subunits are found to behave differently in some cases. The Fe-His(F8) bonds in the α subunits of nitrosohemoglobin are cleaved upon the structural change by the addition of IHP, while those in β subunits remain unchanged (Perutz et al., 1976). In going from the deoxy-Ω to deoxy-T structure, differences in behavior of the Fe-His(F8) bonds between α and β subunits are also observed by resonance Raman Fe-Nt(HisF8) stretching frequency and 1H NMR resonance of N3H proton of the proximal His. The resonance Raman Fe-Nt(HisF8) stretching frequency in the α subunits changes from 222 cm⁻¹ to 205 cm⁻¹, while that in the β subunits changes from 224 cm⁻¹ to 218 cm⁻¹ (Nagai and Kitagawa, 1980). On the other hand, the 1H NMR resonance of N3H proton of the proximal His in the α subunits changes from 72 to 60 ppm, while that in the β subunits does not change (Nagai et al., 1982).

During recent years, we have investigated the properties of metal-substituted Hbs α(M)β(Fe₂) and α(Fe)β(M)₂ using the first transition metal ions (M). This metal substitution method is useful in examining the role of Fe (or metal)-His bonds. Since each metal ion has different electronic configurations and natures, the interaction between the metal ion and the proximal His is expected to be different from each other. Thus, one can estimate the role of Fe (or metal)-His bonds in determining the protein conformation by comparison of the properties of metal hybrids. Among metal ions, in the case of CuPP, VOPP, and NiPP, the bond between the metal ion and the proximal His in the α subunits was found to be cleaved upon the conformational change. In these cases, α(Ni)β(Fe)₂ has been extensively studied by various spectroscopic techniques, including x-ray crystallographic analysis (Shibayama et al., 1986a, 1987; Luisi et al., 1990). These features of the Fe (or metal)-His bond in the α subunits led us to consider the heme substitution in the α subunits with protoporphyrin IX (PP) which is incapable of forming the

* The abbreviations used are: Hb, hemoglobin; HbA, human adult hemoglobin; M-Fe hybrid, hybrid Hb in which iron ions in either the α or β subunits are substituted with other metal ions (M); Semihemoglobin α, hybrid Hb in which the β subunits do not carry the heme; Semihemoglobin β, hybrid Hb in which the α subunits do not carry the heme; PP, protoporphyrin IX; NiPP, nickel(II) protoporphyrin IX; CuPP, copper(I) protoporphyrin IX; VOPP, oxovanadium(IV) protoporphyrin IX; IHP, inositol hexaphosphate; CO, carbon monoxide; bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; ppm, parts/million.

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2 N. Shibayama and H. Morimoto, unpublished results.
Fe-His(F8) bond. Although α(PP)β(Fe)2 hybrid appears to have a significant bearing on the interpretation of cooperative behavior of Hb, its functional properties have not been studied for more than 15 years (Leontidou et al., 1974). In addition, we have paid attention to the fact that there is no case where the Fe (or metal)-His bonds are cleaved in the β subunits and focus our interest on the properties of the hybrid Hb in which the Fe-His(F8) bonds are absent in the β subunits. Parkhurst et al. (1970) reported stopped-flow experiments on α(Fe)β(PP)2 and found that the slow phase of the CO binding was characteristic of deoxy-Hb. However, detailed information on the properties of this hybrid, including the pH and IHP effects, are still uncertain. In order to clarify these questions, we report here on the oxygen binding and structural properties of PP-Fe hybrid Hbs.

EXPERIMENTAL PROCEDURES

Preparation of PP-Fe Hybrid Hbs—Protoporphyrin IX was obtained from Sigma. Purity was confirmed by thin layer chromatography on Silica Gel G developed with chloroform/methanol/formic acid/ethanol/water (25:10:1:5:2 v/v). Hbs and its isolated chains were prepared from hemoglobin in carbon monoxide dimer equilibrium scheme because the elution volume of a 2.5% dimer in 0.1 M Tris buffer with 0.1 M chloride could not be analyzed on the basis of the simple tetramer-dimer equilibrium systems such as the present two heme-containing hybrid Hbs. Hill plots become linear at both saturation extremes and the y axis intercepts of low and high saturation asymptotes indicate log K1 and log K2, respectively, where K1 and K2 are Adair constants of the first and second oxygen binding reactions, respectively. The values of the Adair constants (K1 and K2), the oxygen affinity (P50), and Hill’s coefficient (nmax) are listed in Table 1.

RESULTS

Oxygen Equilibrium Curves for PP-Fe Hybrid Hbs

As neither prosthetic group-free subunits nor PP-containing subunits bind oxygen, the oxygen equilibrium properties of semiHb hybrids and Fe-PP hybrids are entirely derived from two heme-containing subunits. The Hill plot, log Y/(1 - Y) versus log P, where Y is a fraction of oxygenated species, (1 - Y) is a fraction of deoxygenated species, and P is a partial pressure of oxygen is a convenient expression of the oxygen equilibrium curves, to determine empirical constants such as P50 (half-saturation pressure of oxygen) and nmax (Hill’s coefficient for cooperativity). In two-step oxygen equilibrium systems such as the present two heme-containing hybrid Hbs, Hill plots become linear at both saturation extremes and the y axis intercepts of low and high saturation asymptotes indicate log K1 and log K2, respectively, where K1 and K2 are Adair constants of the first and second oxygen binding reactions, respectively. The values of the Adair constants (K1 and K2), the oxygen affinity (P50), and Hill’s coefficient (nmax) are listed in Table 1.

Fig. 1 shows Hill plots of oxygen equilibrium curves of hybrid Hbs containing hemes in the β-subunits, namely, α(-)β(Fe)3 and α(PP)β(Fe)2 at pH 7.4. α(-)β(Fe) exhibited a high affinity for oxygen (P50 = 1.2 torr) and little cooperativity (nmax = 1.1). The effects of pH and IHP on its oxygen affinity were negligible (data are not shown). α(PP)β(Fe)2 on the other hand, exhibited a very low oxygen affinity at all measured pH, as compared with those of native HbA (Fig. 2); the low-saturation asymptote (or K1), of α(PP)β(Fe)2 even at high pH (pH 9.0) was comparable to that of native HbA at pH 6.5. The low saturation asymptote at pH 6.5, on the other hand, was nearly equivalent to that of native HbA at pH 7.4 in the presence of IHP. Log P50 and nmax of α(PP)β(Fe)2 are plotted as a function of pH in Fig. 3A. The effects of IHP and pH on its oxygen affinity were small in comparison with those of native HbA (Imai, 1982). Hill’s coefficient of α(PP)β(Fe)2 (nmax = 1.0-1.1) was independent of pH over the pH range examined, suggesting the weak cooperative interaction between two β subunits. The proton release associated with the first oxygenation step was measured from the slope of the log K1 versus pH plot at pH 7.4, was estimated to be 0.16 proton/O2. The concentration dependence of oxygen affinity was observed in the range from 15 to 60 μM on a tetramer basis (Table 1), indicating that the influence of the tetramer-dimer equilibrium on the results was small. Gel filtration experiments on α(PP)β(Fe-CO)2 were carried out to obtain information on the tetramer-dimer equilibrium. The results could not be analyzed on the basis of the simple tetramer-dimer equilibrium scheme because the elution volume of a 2.5

\[ \alpha(-)β(Fe) \] and \( α(Fe)(β) \) applied in the present study are considered mainly as a dimer system (Cassory and Banerjee, 1971).
TABLE I

| pH   | IHP | \( P_0 \) | \( n_{max} \) | \( K_d \) | \( K_I \) |
|------|-----|---------|-------------|-----------|-----------|
| 6.5  | -   | 128     | 1.05        | 0.00689   | 0.00869   |
|      | -   | (122)   | (1.07)      | (0.00717) | (0.00939) |
|      | +   | 197     | 1.07        | 0.00438   | 0.00584   |
| 7.4  | -   | 89.3    | 1.10        | 0.00923   | 0.0133    |
|      | -   | (106)   | (1.05)      | (0.00847) | (0.0103)  |
|      | +   | 190     | 1.06        | (0.00471) | (0.00585) |
| 9.0  | -   | 49.5    | 1.00        | 0.0199    | 0.0205    |
|      | -   | (58.7)  | (1.04)      | (0.0162)  | (0.0180)  |
|      | +   | 111     | 1.02        | 0.00668   | 0.00932   |

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1. In the presence of IHP (2 mM).
2. In the absence of IHP.
3. Oxygen pressure (torr) at half-saturation.
4. Maximum slope of the Hill plot.
5. Adair constant for the first oxygen binding to the ferrous subunits of the hybrid Hbs (torr-1).
6. Oxygen pressure (torr) at half-saturation.
7. Adair constant for the second oxygen binding to the ferrous subunits of the hybrid Hbs (torr-1). The tetramer-dimer equilibrium may affect \( K_d \) values more significantly than \( K_I \) values.
8. Protein concentration; 30 nM (tetramer).

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**FIG. 1.** Hill plots of oxygen equilibria at pH 7.4 for \( \alpha(-)\beta(Fe)_{2} \) (A) and \( \alpha(PP)\beta(Fe)_{2} \) (C). Y is fractional oxygen saturation of ferrous subunits; \( P \) is partial pressure of oxygen in Torr. Filled symbols denote samples with 2 mM IHP. Conditions are as follows: in 50 mM Tris with 100 mM chloride; protein concentration, about 15 pM (tetramer); measurement temperature, 25 °C; wavelength of detection light, 560 nm.

**FIG. 2.** Hill plots of oxygen equilibria for native HbA (solid line) and \( \alpha(PP)\beta(Fe)_{2} \) (O, A) at various pH values. The buffer system applied is 50 mM Bis-Tris or Tris with 100 mM chloride. Other experimental conditions are described in the legend to Figure 1.

**FIG. 3.** pH dependence of \( \log P_0 \) and \( n_{max} \) for PP-Fe hybrid Hbs: (A) \( \alpha(PP)\beta(Fe)_{2} \); (B) \( \alpha(Fe)\beta(PP)_{2} \). Filled symbols indicate the presence of IHP (2 mM). Protein concentration is 15 nM (tetramer). For \( \alpha(Fe)\beta(PP)_{2} \), PP was added into Hb samples to make its total concentration 45 nM, including the bound PP in the \( \beta \) subunit.

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In addition, \( \alpha(Fe)\beta(PP)_{2} \) apparently showed a high cooperativity (\( n_{max} = 1.3-1.4 \)) above pH 7.4 in the absence of IHP. Change in \( P_0 \) was found to be small over a protein concentration range between 15 and 30 nM on a tetramer basis (Table I). The oxygen affinity of \( \alpha(Fe)\beta(PP)_{2} \) exhibited a significantly large pH dependence (Fig. 3B).
positions were shifted downfield by 0.3 ppm. In addition to the signal shifts, the 14 ppm resonance markedly reduced its intensity, while the intensity of the 11 ppm resonance remained unchanged. In the presence of IHP, the recovery in shown in Fig. 5A, these T marker signals concentration, about 15 μM (tetramer); total protoporphyrin of CO to its ferrous subunits at pH 7.4, their resonance observed at 13.8 and 10.8 ppm in full intensity. Upon ligation of metal substituted hybrid Hbs as T- or R-state (Simolo et al., 1986). Hydrogen-bonded proton resonances (A) and ring current shifted resonances (B). Conditions: in 50 mM Bis-Tris or Tris with 100 mM chloride at 25 °C. A symbol "T" represents the T-state marker.

**NMR Measurements**

**Hydrogen-bonded Proton Resonances**—Fig. 5 illustrates the 1H NMR spectra of native HbA and α(PP)β(PP)2. Upon deoxygenation, native Hb exhibits exchangeable proton resonances at about 14 and 11 ppm, which are not observed for the liganded native Hb (Fig. 5A). The resonances at 14 ppm were assigned to the hydrogen bond at the αβ interface between α,βTyr and β,βAsp (Fung and Ho, 1975). The validity of the assignment was confirmed by a nuclear Overhauser effect investigation (Rusu et al., 1987). On the other hand, the resonance at 11 ppm was assigned to the hydrogen bond at the αβ interface between α,βAsp and β,βTrp by site-directed mutagenesis techniques (Ishimori et al., 1992). These two signals have been used to define the quaternary structure of metal substituted hybrid Hbs as T- or R-state (Simolo et al., 1985; Ishimori and Morishima, 1988; Inubushi et al., 1986). The corresponding resonances for deoxy-α(PP)β(PP)2 were observed at 13.8 and 10.8 ppm in full intensity. Upon ligation of CO to its ferrous subunits at pH 7.4, their resonance positions were shifted downfield by 0.3 ppm. In addition to the signal shifts, the 14 ppm resonance markedly reduced its intensity, while the intensity of the 11 ppm resonance remained unchanged. In the presence of IHP, the recovery in the intensity of the resonance at 14 ppm was observed. As shown in Fig. 5A, these T marker signals for the liganded form were little affected by pH variation between pH 6.5 and 7.4. As the pH was raised to 9.0, however, the lower field T-state marker disappeared completely. On the other hand, the higher field T-state marker was shifted upfield and reduced its intensity. Fig. 6 shows the 1H NMR spectra of native Hb A, deoxy-semiHb α, and α(PP)β(PP)2. Unlike deoxy-semiHb α, the deoxygenated form of α(PP)β(PP)2 showed both of the exchangeable proton resonances at 14 and 11 ppm (Fig. 6A). Their signal intensities were comparable to those of the corresponding signals observed in deoxy native Hb. The ligation of CO to the α(Fe) subunits significantly reduced the intensity of the lower field T-state marker, which was observed at 13.7 ppm, and the resonance at 11 ppm was completely disappeared. It was found that IHP increased the intensity of the lower field T-state marker. In addition, the higher field T-state marker was restored as a small shoulder at 10.8 ppm.

**Ring Current-shifted Resonances**—The upfield region includes resonances related to the diamagnetic anisotropies of
a porphyrin ring. As shown in Fig. 5B, HbCO exhibits the resonance at -2.0 ppm which has been assigned to the $\gamma_{2}$-methyl of E11-Val in both $\alpha$(Fe-CO) and $\beta$(Fe-CO) subunits (Lindstrom et al., 1972; Lindstrom and Ho, 1978; Dalvit and Ho, 1985). In contrast, $\alpha$(PP)$_2$($\beta$Fe$_2$Co$_2$) showed a more complicated signal pattern at pH 7.4 in this region (Fig. 5B). Assignment of the resonance of E11-Val was derived from comparison of the spectra between deoxy $\alpha$(PP)$_2$($\beta$Fe$_2$Co$_2$) and $\alpha$(PP)$_2$($\beta$Fe$_2$Co$_2$)$_2$ at pH 7.4. Since deoxy Fe-subunits do not show the resonance of the E11-Val, the resonances observed between 3.0 and 4.5 ppm for deoxy-$\alpha$(PP)$_2$($\beta$Fe)$_2$ were considered to originate from $\alpha$(PP) subunits. Thus, the resonance at 2.4 ppm was attributable to the $\gamma_{2}$-methyl of E11-Val in $\beta$(Fe-CO) subunits. It should be noted that the resonance position of the E11-Val in $\beta$(Fe-CO) subunits was dependent upon solution conditions as shown in Fig. 5B. In the presence of IHP, the resonance of E11-Val was observed at -2.6 ppm. At lower pH, this signal was situated at the position almost identical to that observed in the presence of IHP. As the pH was raised, the position of this signal was shifted downfield and approached that of the E11-Val resonance in HbCO. The unassigned resonances originating from $\alpha$(PP) subunits exhibited the pH dependence as well, except a resonance at about 3.9 ppm. The 'H NMR spectra in the upfield region of native HbCO and $\alpha$(FeCoPP) are shown in Fig. 6B. In deoxy $\alpha$(Fe)$_2$Fe$_2$Co$_2$, $\alpha$(PP)$_2$($\beta$Fe)$_2$ subunits exhibited a signal pattern different from that observed in $\alpha$(PP) subunits. Upon CO ligation, $\alpha$(Fe)$_2$Fe$_2$Co$_2$ in $\alpha$(Fe)$_2$Fe$_2$Co$_2$ showed a resonance of the E11-Val at about -1.9 ppm, as in the liganded native HbA. Unlike its complementary hybrid Hb, the resonance of the E11-Val did not shift even in the presence of IHP.

**DISCUSSION**

The present work has demonstrated that deoxy-Hb tetramer, upon replacement of protohemes in one type of subunits with PPs, retains its conformation in a deoxy quaternary state having the Fe-containing subunits with low oxygen affinity. Thus, PP behaves like deoxy heme in these hybrids, even though no metal-proximal His bond is present in the PP-Fe-Ru(CO) (Ishimori and Morishima, 1989) hybrid Hbs. Though no metal-proximal His bond is present in the PP-Fe-Ru(CO) hybrid Hbs, these are compared with those of Fe-PP hybrids and $\alpha$(M)$_2$ subunits of the $\alpha$-substituted hybrids may be described as Co > (Fe), Ni > PP, as in the case of the globin-free system. This order coincides well with that of the $K_1$ values in the $\beta$(Fe$_2$Co$_2$) subunits of these hybrids. The data in Table II allow us to conclude that the strength of the interaction between the metalloporphyrin and the proximal His in the $\alpha$ subunit is linked to the affinity of the $\beta$ subunits for the first oxygen. On the other hand, the examination of the $\beta$-substituted hybrids indicates that the nature of (metallo)porphyrin or the presence and absence of the metal-His bonds in the $\alpha$(M)$_2$ subunits does not seem to significantly affect either the $K_1$ values or heterotropic responses, such as pH and IHP effects, of the $\alpha$(Fe)$_2$Fe$_2$Co$_2$ in these $\alpha$(Fe)$_2$Fe$_2$Co$_2$ hybrids at least Co, Fe, Ni, and PP are concerned. Indeed, $\alpha$(Fe)$_2$Fe$_2$Co$_2$ behave like FeHb, indicating that the strength of the interaction between the porphyrin and the proximal His in the $\beta$ subunit is slightly linked to the first oxygen binding to the $\alpha$ subunit.

Proton NMR spectra of deoxy-HbA and deoxy-Fe-PP hybrids (Figs. 5 and 6) exhibit exchangeable proton resonances at 14 and 11 ppm, which have been assigned to the hydrogen bonds present in the deoxy quaternary structure. The 14 ppm resonance has been identified to the intersubunit hydrogen bond between $\alpha_{4}$-$\gamma$Tyr and $\beta_{59}$Asp (Fung and Ho, 1975), whereas the 11 ppm resonance was attributed initially to the intrasubunit hydrogen bond between $\beta_{4}$Val and $\beta_{4}$Tyr (Viggiano et al., 1978) and has been subsequently reassigned to the intersubunit hydrogen bond between $\alpha_{4}$Asp and $\beta_{7}$Tyr (Ishimori et al., 1992). The presence of these two structural markers in deoxy-HbA and deoxy-Fe-PP hybrids confirms that these compounds are in the deoxy quaternary state in solution. The absence of these two structural markers in deoxy semi Hb $\alpha$ (Fig. 6A) is consistent with the notion that semiHb $\alpha$ is in a dimeric state (Cassory and Banerjee, 1971). Upon ligation of CO to the $\beta$(Fe)$_2$ subunits at pH 7.4, the intensity of the 14 ppm resonance was reduced significantly without affecting the 11 ppm resonance in $\alpha$(PP)$_2$($\beta$Fe$_2$Co$_2$) (Fig. 5) and $\alpha$(Ni)$_2$($\beta$Fe$_2$Co$_2$) (Shibayama et al., 1987). By contrast, both 14 and 11 ppm resonances in $\alpha$(Co)$_2$($\beta$Fe$_2$Co$_2$) are replaced by the 10.7 ppm resonance which has served as a structural marker for the oxy quaternary structure (Fung and Ho, 1975). These observations suggest that both di-ligated $\alpha$(PP)$_2$($\beta$Fe$_2$Co$_2$) and $\alpha$(Ni)$_2$($\beta$Fe$_2$Co$_2$) are still in a deoxy quaternary state, whereas the di-ligated $\alpha$(Co)$_2$($\beta$Fe$_2$Co$_2$) has shifted to the oxy quaternary state. The observed changes in the hydrogen-bonded NMR spectra, which were induced by ligation and pH changes, may be derived from either actual breakage/formation of specific hydrogen bonds or the changes in the exchange rate of protons between hydrogen-bonded protons and solvent water protons, which were caused by local structural alterations. Thus, the observed asynchronous changes in the structural markers may indicate the presence of a series of structural substates within the deoxy quaternary state, ranging from the low affinity substate with diminished homotropic and heterotropic effects on one hand, to the
substate immediately adjacent to the oxy quaternary state on the other. Asynchronous changes in the hydrogen-bonded NMR spectra, as reported here, have been frequently observed in a wide variety of hybrid Hbss upon partial ligation (Miura and Ho, 1982, 1984; Shibayama et al., 1987; Inubushi et al., 1986; Ishimori et al., 1991), indicating that the presence of several substates in the deoxy quaternary structure is a generally observable phenomenon.

These NMR results indicate that \( \alpha(PP)_{2}\beta(Fe)_{2} \) and \( \alpha(Ni)_{2}\beta(Fe)_{2} \) are strongly constrained to the deoxy quaternary structure, which is less affected by the ligation of CO to the \( \beta(Fe)_{2} \) subunits, in comparison with \( \alpha(CO)_{2}\beta(Fe)_{2} \), which shifts to the oxy quaternary state upon ligation. Thus, the pattern of changes in the hydrogen-bonded NMR spectra is consistent with the order of \( K_{i} \) values of the \( \beta(Fe)_{2} \) subunits in these metal hybrids. On ligation of CO to the \( \alpha(Fe)_{2} \) subunits, the drastic change in the hydrogen-bonded resonance region was observed for \( \alpha(Fe)_{2}\beta(PP)_{2} \), \( \alpha(Fe)_{2}\beta(Ni)_{2} \), \( \alpha(Ni)_{2}\beta(Fe)_{2} \) (Shibayama et al., 1987), and \( \alpha(Fe)_{2}\beta(CO)_{2} \). However, the extent of the structural change observed at the \( \alpha_{2}\beta_{2} \) interface is relatively smaller for \( \alpha(Fe-CO)_{2}\beta(PP)_{2} \) than for \( \alpha(Fe-CO)_{2}\beta(Ni)_{2} \) and \( \alpha(Fe-CO)_{2}\beta(CO)_{2} \). Conversely, in the presence of 2 mM IHP, the local environment at the \( \alpha_{2}\beta_{2} \) interface of \( \alpha(Fe-CO)_{2}\beta(PP)_{2} \) was somewhat perturbed upon ligation, while \( \alpha(Fe-CO)_{2}\beta(Ni)_{2} \) and \( \alpha(Fe-CO)_{2}\beta(CO)_{2} \) showed no change at this region (Shibayama et al., 1987). Such structural differences among \( \beta \)-substituted hybrids appear to be minor in comparison with the case for \( \alpha \)-substituted hybrids. In Fe-PP and Fe-Ni hybrids, the ligand-induced structural changes are more pronounced upon ligation to the \( \alpha(Fe)_{2} \) subunits than to the \( \beta(Fe)_{2} \) subunits. However, the opposite case was found to be Fe-Co hybrids (Inubushi et al., 1986). Probably the reason for the essential difference is that CoPP in the \( \alpha \) subunit makes the deoxy tetramer much less constrained than PP or NiPP in the \( \alpha \) subunit.

Ring-current-shifted NMR spectra of the \( \alpha(Fe-CO)_{2} \) subunits of \( \alpha(PP)_{2}\beta(Fe-CO)_{2} \) and \( \alpha(Fe-CO)_{2}\beta(PP)_{2} \) exhibit significant subunit inequivalence. The ring-current-shifted E11 Val \( \gamma_{2} \) methyl resonance of the \( \alpha(Fe-CO)_{2} \) subunits of \( \alpha(Fe-CO)_{2}\beta(PP)_{2} \) is observed at \(-1.9 \) ppm independent of the quaternary state and solution conditions (Fig. 6). The \( \beta(Fe-CO)_{2} \) subunits of \( \alpha(PP)_{2}\beta(Fe-CO)_{2} \), on the other hand, show the resonance at \(-2.4 \) ppm at pH 6.5 in the deoxy quaternary state (Fig. 5). This resonance shifts toward \(-2.1 \) ppm as pH is raised and/or the quaternary structure shifts toward the oxy-like structure. The upfield-shifted resonance of the \( \beta(Fe-CO)_{2} \) subunits is derived from a stronger interaction of E11 Val with the porphyrin ring in the \( \beta(Fe-CO)_{2} \) subunits in the deoxy quaternary state than in the oxy quaternary state. In fact, x-ray studies indicated the E11 Val \( \gamma_{2} \) methyl is located closest to the heme plane in deoxy Hb (Fermi, 1975). The previous observations on the ring-current-shifted resonances of low affinity \( \alpha(Ni)_{2}\beta(Fe)_{2} \) (Shibayama et al., 1987), Hb Kansas (\( \beta^{202} \) Asn \( \rightarrow \) Thr), and HbM Iwate (\( \alpha^{20} \) His \( \rightarrow \) Tyr) (Ogawa et al., 1972; Mayer et al., 1973) are consistent with our present interpretation. Thus, the resonance of \( \alpha(PP)_{2}\beta(Fe-CO)_{2} \) observed at \(-2.4 \) ppm suggests that the distal environment of the \( \beta(Fe-CO)_{2} \) subunits in \( \alpha(PP)_{2}\beta(Fe-CO)_{2} \) is in a deoxy tertiary state and consequently the ligated \( \alpha(PP)_{2}\beta(Fe-CO)_{2} \) is considered to be in the deoxy quaternary state at pH 7.4.

The present work on the Fe-PP hybrid Hbs provides important clues to understand the critical role of the Fe-His bonds in controlling the ligand affinity and allostericity of Hb. The \( K_{i} \) values of \( \alpha(PP)_{2}\beta(Fe)_{2} \) are much smaller and much less influenced by pH or IHP than those of Hb, indicating that deoxy \( \alpha(PP)_{2}\beta(Fe)_{2} \) is strongly biased to the deoxy quaternary structure. On the other hand, the \( K_{i} \) values and the sensitivity to pH and IHP of \( \alpha(Fe)_{2}\beta(PP)_{2} \) are similar to those of deoxy Hb, indicating both are similarly constrained toward the deoxy quaternary state. This shows that metal-free protoporphyrin IX effectively mimics deoxy heme to induce the deoxy quaternary structure in these hybrids. This feature is stronger in the \( \alpha \)-substituted hybrid. This further implies that Hb assumes preferentially the deoxy quaternary structure in the absence and/or weakening of the Fe-His bonds, partially in the \( \alpha \) subunits. Furthermore, this notion is consistent with the known oxygenation characteristics of two naturally occurring Hb mutants, in which the heme environment in the \( \alpha \) subunits is altered. The present results on \( \alpha(PP)_{2}\beta(Fe)_{2} \) may offer a rational answer to the puzzling question regarding the unusual oxygenation characteristics of HbM Taiwate (\( \alpha^{20} \)His \( \rightarrow \) Tyr) (Kikuchi et al., 1964; Hayashi et al., 1966) and HbM Boston (\( \alpha^{20} \)His \( \rightarrow \) Tyr) (Suzuki et al., 1965). These two HbMs, having the His \( \rightarrow \) Tyr mutation in the \( \alpha \) subunits, exhibit very low oxygen affinities and substantially diminished Bohr effect, in the manner similar to \( \alpha(PP)_{2}\beta(Fe)_{2} \), as reported here. The distal and proximal structures of the heme sites in the \( \alpha \) subunits of HbA, \( \alpha(PP)_{2}\beta(Fe)_{2} \), HbM Iwate, and HbM Boston are schematically compared in Fig. 7. In HbM Iwate, F8 His is replaced by Tyr, so that the distance (d in Fig. 7) between the porphyrin plane and the \( \alpha \) carbon of the F8 residue is extended in comparison with that in HbA. In HbM Boston, the 6th coordination of the heme iron is shifted from the F8 His to the E7 Tyr (Pulsinelli et al., 1973), resulted in no bonding between the heme iron and the F8 His, just as is found in the \( \alpha \) subunits of \( \alpha(PP)_{2}\beta(Fe)_{2} \). In the absence of the Fe-F8 His bonding or the absence of the structural constraints by the Fe-proximal His bond, the \( \alpha \) carbon of the F8 His in the F helix would freely shift to an energetically more stable position in the deoxy quaternary state. As a consequence, the distance between the porphyrin plane and the \( \alpha \) carbon of the F8 residue in the \( \alpha \) subunits would be extended in \( \alpha(PP)_{2}\beta(Fe)_{2} \) and HbM Boston, in comparison with that of HbA. What is the structural feature common among these three low affinity Hb variants? The nature of the proximal and/or distal residues, the presence and absence of the porphyrin metal ion, and the oxidation state of the porphyrin

| TABLE II | Comparison of \( K_{i} \) values among PP-Fe, Ni-Fe, and Co-Fe hybrid Hbs |
|-----------------|-----------------|-----------------|
| \( K_{i} \) of \( \beta \)-Fe subunits** (torr**-1)** | \( K_{i} \) of \( \alpha \)-Fe subunits (torr**-1)** | \( K_{i} \) of HbA (torr**-1)** |
| PP | Ni* | Co* | PP | Ni* | Co* | Fe |
| pH 7.5 | 0.0111 | 0.0227 | 0.270 | 0.0200 | 0.0300 | 0.0400 | 0.0476 (pH 7.45) |
| pH 8.5 | 0.0368 | 0.0617 | 1.272 | 0.0950 | 0.122 | 0.116 | 0.123 |

* Estimated values of \( K_{i} \) are shown.
* Data were taken from Shibayama et al. (1986a).
* Data were estimated from Tsubaki and Nagai (1979).
metal ion are not shared among these three low affinity Hb variants, α(PP)β(Fe)₂, HbM Iwate, and HbM Boston. The presence of metal-free PP and an extended distance between the porphyrin plane and the α carbon of the F8 residue in the α subunits appear to be a common denominator. From this point of view, it is tempting to suggest a possible linkage between the $K_1$ value of the $β(Fe)₂$ subunits in the α-substituted hybrid and the distance (or position) of the proximal His relative to the porphyrin plane in the α subunits.

The tertiary structural constraint of the heme-proximal His system in deoxy-Hb has been interpreted in terms of the stretching and/or tilt of the Fe-His bond, which can be monitored by electronic absorption, resonance Raman Fe-His stretching mode, and hyperfine-shifted NaH proton NMR of the proximal His (Sugita, 1975; Matsukawa et al., 1979; Nagai and Kitagawa, 1980; Nagai et al., 1982). However, the low affinity state of HbM Iwate which has a longer F8 side chain cannot be explained by the assumption of the localized constraint (the stretching and/or tilt) in the 5th axial bond of the α heme group. As mentioned above, a more logical explanation may be that an extended distance and/or position of the α carbon of the F8 residue relative to the porphyrin plane plays a vital role in modulating properties of the deoxy quaternary structure. The fact that deoxy-α(PP)β(Fe)₂ and deoxy-HbM Boston assume a deoxy quaternary state which has a very low oxygen affinity and a diminished Bohr effect suggests that this deoxy quaternary state may be an energetically more stable and preferred state for deoxy-Hb tetramers in general. Deoxy-HbA may be prevented from shifting to this deoxy quaternary state by the presence of the Fe-proximal His bonds in the α subunits. In other words, the Fe-proximal His bonds in the α subunits act as a constraint against the shift of Hb to the energetically more stable deoxy quaternary state and keep Hb in energetically less stable deoxy quaternary states which are homotropically and heterotropically sensitive to allosteric effectors and which can readily and reversibly shift to the oxy quaternary state upon ligation. Deoxy-HbM Iwate, which has the Fe-proximal His bonds in the α subunits, accomplishes the shift to the energetically more stable deoxy quaternary state by having a larger F8 residues (His versus Tyr). Thus, the Fe-proximal His bonds in the α subunits may play two roles: the one as the conduit of the ligand-induced tertiary structural change (Perutz, 1970) and another as the structural constraint to keep Hb in physiologically important substrates and to prevent the structural shift to an energetically more stable deoxy quaternary state, which is represented by α(PP)β(Fe)₂ and HbM Boston. Perutz (1970) put forward an elegant hypothesis that upon release of the bound ligand the axial out-of-plane movement of the heme Fe toward the proximal side pushes proximal His, which in turn imparts a shift of the F helix, leading to the shift of the αβ₂ subunit interface to the deoxy quaternary structure. However, the fact that the Fe-His bond in the α subunits is stretched from 2.0 to 2.2 Å upon shifting from the deoxy-R state to the deoxy-T state (Perutz et al., 1987), that the metal-proximal His bond is broken in the α subunits of deoxy-α(Ni)β(Fe)₂, and of HbANO in the presence of IHP, and that α(PP)β(Fe)₂ assumes a deoxy quaternary structure are difficult to explain by this push action of the heme iron, as proposed by Perutz (1970). They may be better explained on the basis of the structural behavior on the proximal side (the F helix and FG corner) of the α subunits.

The situation for the β-substituted hybrids is less clear. Spectroscopic and x-ray crystallographic data show no obvious structural distortion in the prosthetic group and its environment in the β subunits in deoxy-HbA and metal-substituted hybrids except for the steric hindrance exerted by the closely positioned E11 Val residue, as revealed by x-ray and ring-current-shifted NMR data. The present work on α(Fe)β(PP)₂ shows that even in the absence of the Fe-proximal His bonds in the β subunits, deoxy-α(Fe)β(PP)₂ takes a deoxy quaternary state which is homotropically and heterotropically sensitive to allosteric effectors (Fig. 4), in a manner similar to that of deoxy HbA. We suggest that the affinity for the first oxygen ($K_1$) for α(Fe)β(PP)₂ may be regulated by the position of E11 Val in the β subunits relative to the porphyrin plane, since this residue could occupy the ligand-binding site of the β subunits. This notion may explain similarities of $K_1$ values among different β-substituted hybrids, α(Fe)β(PP)₂, α(Fe)β(Ni)₂, and α(Fe)β(β(Fe)₂.

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