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Ruthenium–Iron Hybrid Hemoglobins as a Model for Partially Liganded Hemoglobin: NMR Studies of Their Tertiary and Quaternary Structures†

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ABSTRACT: Diruthenium-substituted Ru–Fe hybrid hemoglobins (Hb) were synthesized by heme substitution from protoheme to ruthenium(II) carbonyldeuteroporphyrin in the α or β subunits. As the carbon monoxide coordinated to ruthenium(II) is not released under physiological conditions, deoxygenated Ru–Fe hybrid derivatives [$\alpha(Fe)_2\beta(Ru–CO)_2$ and $\alpha(Ru–CO)_2\beta(Fe)_2$] can serve as models for half-liganded Hbs. On the basis of proton NMR spectra of hyperfine-shifted proton resonances, these Ru–Fe hybrid Hbs have only small structural changes in the heme environment of the partner subunits at low pH. The proton NMR spectra of the intersubunit hydrogen-bonded protons also showed that the quaternary structures of the two complementary hybrids both remain in the "T-like state" at low pH, suggesting that the T to R structural conversion is induced by ligation of the third ligand molecule. Marked conformational changes in the heme vicinity are observed at high pH only for $\alpha(Ru–CO)_2\beta(Fe)_2$, and its quaternary structure is converted into the "R state"; the $\alpha(Fe)_2\beta(Ru–CO)_2$ hybrid does not undergo this change. This implies that the free-energy difference between the two quaternary states is smaller in the α-liganded hybrid than in the β-liganded one.

Hemoglobin (Hb) has long served as a paradigm for cooperative ligand binding in proteins (Antonini at al., 1971; Edelstein, 1975; Dickerson & Geis, 1983). On the basis of numerous experimental studies, particularly X-ray crystallography (Perutz, 1976, 1979) and nuclear magnetic resonance (NMR) spectroscopy (Ho et al., 1975; Shulman et al., 1975), a number of specific mechanisms to account for the cooperativity have been proposed (Monod et al., 1965; Koshland et al., 1965; Gelin & Karplus, 1977; Warshel, 1977; Perutz, 1976). However, detailed understanding of the control mechanism of ligand affinity in Hb may well be achieved only by analyzing the tertiary and quaternary structures and functional properties of the protein as a function of the degree of ligation. Therefore, the most uncompromising problem that we encounter in studying the quaternary and tertiary structural change induced by ligation is physical and chemical characterizations of Hb species at the intermediate state of ligation, which have been very elusive owing to the difficulty in isolating such species.

To gain an insight into structural characterizations of the partially liganded Hb, the half-liganded Hbs have been studied by utilizing valency hybrid Hb (Ogawa & Schulman, 1972), metal hybrid Hb (Inubushi et al., 1983, 1986; Blough et al., 1980, 1982; Simolo et al., 1985; Shibayama et al., 1987), and intersubunit cross-linked Hb (Miura & Ho, 1982, 1984). However, there has not been an ideal model which is stable enough to study the structure of partially liganded Hb in detail, which would be requisite in characterizing the effect of partial ligation on the properties of individual subunits and the tetrameric Hb molecule as well. Ogawa and Shulman (1972) used $Fe^{III}–CN^-$ heme as a structural model for an oxyheme, but it was very difficult to deoxygenate the Fe(III)–Fe(II) hybrids due to autoxidation. In this sence, iron–ruthenium symmetric hybrid Hbs in which the α or β subunit contains Ru^{II}–CO porphyrin, with the other subunit having a deoxygenated iron(II) porphyrin, could be ideal models for testing the properties of the intermediate ligand binding state. Ru^{II}–CO porphyrin has the following unique properties. (1)

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Ruthenium(II) (carbonmonoxy)porphyrin is virtually isoelectronic with low-spin iron(II) porphyrins involved in oxy-Hb or carbonmonoxy-Hb. Furthermore, since Ru^{II}–CO bonding is very stable, Ru(II) does not readily react with exogenous ligands nor release the sixth ligand. (2) Deoxygenated Ru–Fe hybrid Hbs such as $\alpha(Ru–CO)_2\beta(Fe)_2$ and $\alpha(Fe)_2\beta(Ru–CO)_2$ can bind two moleculr oxygens to the iron-containing subunits. This implies that the oxygen binding property of Ru–Fe hybrids can be compared directly with that of native Hb. Because of the diamagnetic property of Ru(II) in half-lianded Ru–Fe hybrid Hbs, the paramagnetic ^1H NMR resonances from deoxygenated Fe(II) subunits can be observed separately. (3) Half-liganded Ru(CO)–deoxy Fe(II) hybrids can be prepared easily from $\alpha(Ru–CO)_2\beta(Fe–O_2)_2$ and $\alpha(Fe–O_2)_2\beta(Ru–CO)_2$ by flushing with Ar gas and addition of dithionite. The $Ru^{II}CO$-containing subunit is therefore expected to be in a "fixed" oxy-like tertiary and quaternary structure, and Ru–Fe hybrid Hbs can serve as models for the intermediate species in the half-liganded state and deserve to be characterized structurally by physical and chemical methods.

^1H NMR is a powerful tool in studies of the tertiary and quaternary structural changes induced by ligand binding to the Hb molecule (Ho & Russu, 1981). NMR studies of Ru–Fe hybrid Hbs may offer an opportunity to monitor these structural alterations in Hb by use of the following characteristic NMR features: (1) the hyperfine-shifted resonance of the imidazole N_1H proton of the proximal histidine (His-F8) coordinated to the paramagnetic heme iron and the resonances of the methyl groups which are attached to the porphyrin skeleton and (2) the exchangeable proton resonances due to inter- and intrasubunit hydrogen bonds associated with the tertiary and quaternary structural changes of the Hb molecule. The hyperfine-shifted proximal histidine N_1H and the heme methyl resonances have been assigned to the Fe-containing subunits (La Mar et al., 1977; Takahashi et al., 1980). Proton resonances in the protein hydrogen-bonded region are associated with the T to R quaternary transition in Hb (Ogawa et al., 1972). The "T-state" and "R-state" markers have been assigned to specific hydrogen bonds in the inter- and intrasubunit interface (Fung & Ho, 1975; Viggiano et al., 1978), and they have been used to monitor the T to R transition in a variety of Hb species (Ho & Russu, 1981). In this paper, we report some unique structural features of these Ru–Fe hybrid Hbs as revealed by ^1H NMR spectroscopy in relation to the tertiary and quaternary structural changes induced by the ligand binding to either α or β subunits. We also discuss some implications for the allosteric mechanism of Hb.

Materials and Methods

Ruthenium(II) carbonyldeuteroporphyrin[1] (Ru-DPIXCO) was prepared by a variant of the previous method (Tsutsui et al., 1971; Morishima et al., 1986a,b).

Hemolysate was prepared in the usual manner from fresh whole blood obtained from the local blood bank. Hb A and its isolated chains were prepared in carbonmonoxide forms as described by Kilmartin et al. and Gerai et al. (Kilmartin & Rossi-Bernadi, 1971; Kilmartin et al., 1973; Gerai et al., 1969). Heme-free chain globins were prepared from isolated α and β chains by the method of Shibayama et al. (1987). The reconstitution of Ru-DPIXCO-containing chains to form the tetrameric hybrid Hb was performed as reported by Shibayama et al. (1987). Apo α chain (500 mg) was dissolved in 300 mL of 20 mM borate/NaOH buffer (pH 12). The spectrophotometric titration of apoglobin with Ru-DPIXCO at 400 nm gave a well-defined inflection point, from which a molecular stoichiometry of 1:1 was estimated. The solution of apo α chain was mixed with a slight excess of Ru-DPIXCO, which was dissolved in a minimal amount of dimethylformamide (DMF). The mixture was gently stirred overnight in the dark and then concentrated by ultrafiltration and passed through a Sephadex G-25 (Pharmacia) column of 20 mM borate/NaOH buffer, pH 10.5. The concentration of the $\alpha(Ru–CO)$ chain was determined from the absorption of the Soret band. An equimolar amount of $\beta(Fe–O_2)$ chains was treated with 32 mM DL-dithiothreitol, added to $\alpha(Ru–CO)$ chains, and left at 0 °C for 2 h. The $\alpha(Ru–CO)_2\beta(Fe–O_2)_2$ solution thus obtained was passed through a Sephadex G-25 column using a 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.2, and then applied to a DE-23 cellulose (Whatman) column followed by a CM-23 cellulose (Whatman) column, both of which were equilibrated with the same 20 mM Tris-HCl buffer. The fraction that passed through both DE-23 and CM-23 cellulose columns was collected, concentrated by ultrafiltration, and stored in liquid nitrogen.

The preparation of $\alpha(Fe–O_2)_2\beta(Ru–CO)_2$ was carried out by the procedure described above, with appropriate constituents, Ru-DPIXCO, apo β chain, and $\alpha(Fe–O_2)$ chain.

For the preparation of Ru-HbCO, we used apo-Hb which had been made from native Hb A by the same procedure mentioned above. The apo-Hb was combined with a slight excess of Ru-DPIXCO, and the reconstituted Ru-HbCO was isolated by the purification procedure described above. The purity of all the samples was checked by isoelectric focusing electrophoresis (Ampholine pH 6.5–9).

Millimolar extinction coefficients were calculated on the basis of the Ru concentration determined with AA-780 flameless atom absorption spectrometer.

Half-liganded samples were prepared by the addition of a minimal amount of sodium dithionite ($Na_2S_2O_4$) under an argon atmosphere. All samples were approximately at 1 mM/tetramer in 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) or 50 mM Tris-HCl buffer containing 0.1 M Cl^-.

^1H NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Hyperfine-shifted NMR spectra were obtained with an 8K data transform of ±36 kHz and a 6.5-μs 90° pulse after the strong solvent resonance in H_2O solution was supressed by a 500-μs low-power pulse. We used a Redfield 2-1-X pulse sequence with a 29.5-μs pulse and 8K data points over a 6-kHz spectral width for recording the exchangeable proton resonances in the subunit interface of Hb. The probe temperature was determined to ±0.5 °C by the temperature control unit of the spectrometer. The volume of the NMR sample was 0.3 mL. Proton shifts were referenced with respect to the water signal, which is 4.8 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 23 °C.

Results

UV–Visible Spectra of Ru-Substituted Hb. Figure 1 shows UV–vis spectra of some of the Ru-substituted Hbs, Ru-HbCO (A), $\alpha(Ru–CO)_2\beta(Fe–O_2)_2$ (B), and $\alpha(Ru–CO)_2\beta(Fe)_2$ (C). The absorption spectra of Ru-HbCO indicated that there was a distinctively larger and sharper Soret peak at 396 nm compared with that of native HbCO. The millimolar extinction coefficients of Ru-HbCO which were determined on the basis

[1] Unfortunately, the synthesis of ruthenium(II) carbonylprotoporphyrin (Ru-PPIXCO) was not successful because the vinyl groups of protoporphyrin were modified during the incorporation reaction of ruthenium(II) into porphyrin.

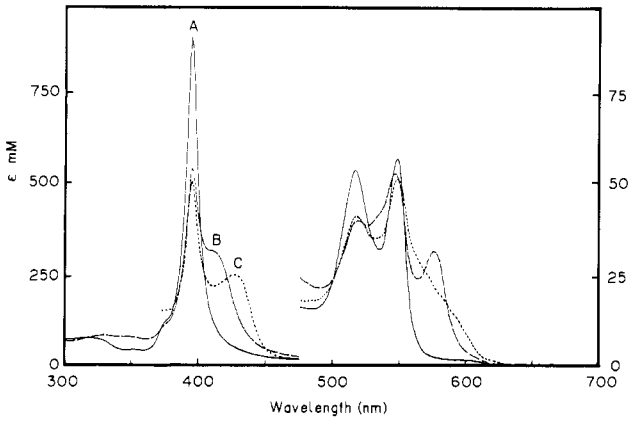

FIGURE 1: Absorption spectra of Ru-HbCO (A), $\alpha(Ru-CO)_2\beta(Fe-O_2)_2$ (B), and $\alpha(Ru-CO)_2\beta(Fe)_2$ (C) in 50 mM Bis-Tris buffer with 0.1 M chloride, pH 7.0, at 25 °C. ϵ (mM) is a millimolar extinction coefficient of Hb (tetramer).

Table I: Spectrophotometric Properties of Ru-Substituted Hbs

(A) Ru-HbCO					
nm	395	517	548		
ϵ (mM/tetramer)	888	54.4	58.0		
(B) $\alpha(Ru-CO)_2\beta(Fe-O_2)_2$					
nm	395	415	519	546	576
ϵ (mM/tetramer)	536	301	39.4	52.2	31.2
(C) $\alpha(Ru-CO)_2\beta(Fe)_2$					
nm	395	427	518	550	
ϵ (mM/tetramer)	499	253	41.5	51.8	
(D) $\alpha(Fe-O_2)_2\beta(Ru-CO)_2$					
nm	395	415	520	545	575
ϵ (mM/tetramer)	545	310	38.1	50.0	30.0
(E) $\alpha(Fe)_2\beta(Ru-CO)_2$					
nm	396	428	517	548	
ϵ (mM/tetramer)	498	256	39.4	48.5	

of the atomic absorption spectra are summarized in Table I. In the oxygenated Ru–Fe hybrid Hb (B), the peaks at 396 and 519 nm are assigned to the subunits which contained Ru(CO), and the shoulder around 415 nm and a peak at the 576 nm are assigned to $Fe(O_2)$ subunits. Deoxygenated Ru–Fe hybrid Hb shows the well-resolved peaks at 396 and 427 nm, arising from α(Ru–CO) subunits and deoxy β(Fe) subunits, respectively. All of these spectral data are compiled in Table I.

Tertiary and Quaternary Structure of Ru-HbCO. Figure 2 shows the ^1H NMR spectra of native HbCO [$\alpha(Fe-CO)_2\beta(Fe-CO)_2$] and Ru-HbCO [$\alpha(Ru-CO)_2\beta(Ru-CO)_2$] in 50 mM Bis-Tris buffer, pH 7.0. For native Hb, the ring current shifted proton peak at −6.6 ppm has been assigned (Lindstrom et al., 1972) to the γ_1-methyl resonance of α and β E11 Val. The corresponding signal for Ru-HbCO was observed at −6.7 ppm with a small shoulder at a downfield side probably due to heme isomerism.[2] In the downfield region, the resonances at 8.2 and 7.4 ppm, which were assigned to the exchangeable protons associated with the $\alpha_1\beta_1$ interface, hydrogen bond between β135 (C1) Tyr and α129 (F9) Asp (Asakura et al., 1976) and hydrogen bond between α103 (G10) His and β108 (G10) Asn (Russu et al., 1987), respectively, and remain almost unchanged upon heme substitution from native heme to Ru-CO heme. A 5.9 ppm resonance, which

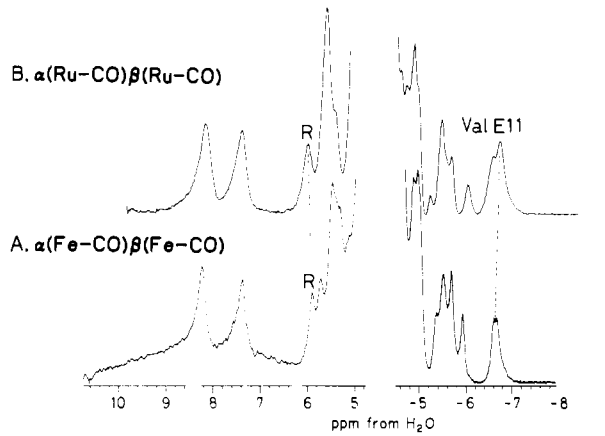

FIGURE 2: Proton NMR spectra (300 MHz) for native HbCO (A) and Ru-HbCO (B) in 50 mM Bis-Tris with 0.1 M chloride, pH 7.0, at 23 °C.

has been utilized as an indicator for the oxy-like quaternary structure (Fung & Ho, 1975), is also insensitive to heme substitution.

Hyperfine-Shifted Proton Resonances. Figures 3A,B and 4A,B show the ^1H NMR spectra of deoxygenated Ru–Fe hybrid Hbs and native deoxy-Hb A which correspond to the half-liganded and unliganded states, respectively. The hyperfine-shifted resonances at 72.1 and 59.5 ppm of the proximal His N_1H have been assigned respectively to the β and α subunits, of native Hb A (Takahashi et al., 1980). The hyperfine-shifted proton resonances of the heme methyl groups were observed in the 5–20 ppm region. The signal at 18.6 ppm was assigned to β subunits, and the resonances at 12.6 and 7.6 ppm were assigned to α subunits (Takahashi et al., 1980). In the spectrum of $\alpha(Ru-CO)_2\beta(Fe)_2$, the hyperfine-shifted resonance at 59.5 ppm for the deoxy α subunit disappeared from this region due to the diamagnetic nature of the porphyrin ruthenium(II) in the α subunits. The N_1H resonance of the β(Fe) subunits which is located at almost the same position as that of native Hb A is slightly shifted downfield with a raise in pH from 6.2 to 8.7. The resonance of the heme methyl group in β(Fe) subunits was also insensitive to the heme substitution of the partner subunits in the low-pH region, while this signal exhibited a marked pH dependence. As the pH is raised, the new signal arising from the heme methyl group appeared at 11.8 ppm, and concomitantly, the peak at 18.6 ppm decreased its intensity.

As Figure 4 shows, the resonance at 58.7 ppm for the counterpart symmetric hybrid Hb, $\alpha(Fe)_2\beta(Ru-CO)_2$, is readily assigned to the proximal His N_1H proton, and those at 12.6 and 7.6 ppm are assigned to the heme methyl groups of the α(Fe) subunits by comparison with deoxy native Hb A. Their signal positions are almost identical with those of deoxy native Hb A and exhibit little pH dependence in the range 5.7–8.2. This observation suggests that the ligation of the β subunits does not affect the heme environmental structure of the α subunits. The positions of the hyperfine-shifted resonances are assembled in Table II.

Intra- and Intersubunit Hydrogen-Bonded Proton Resonances. Upon heme substitution in an α subunit from protoheme to Ru-DPIXCO, the "T-state"[3] signals at 9.2 and 6.1 ppm are observed at almost full intensity at low pH (Figure

[2] For the Ru(CO) heme containing subunit, there are heme orientation isomers as visualized by the splitting of the Val E11 methyl proton resonance. Either of these isomers is not converted to the other in time, because Ru-bound CO is strongly fixed at the heme coordination site. However, it was shown in our previous paper (Ishimori & Morishima, 1986) that the heme disorder in one subunit does not affect the quaternary structure of the complementary subunit and the quaternary structure as well.

[3] We defined the deoxy structure as "T" and the oxy structure as "R" as frequently used in the literature. In this paper we used "T-like" and "R-like" structures when the quaternary structure of Ru–Fe hybrid Hb is close to the deoxy and oxy structures, respectively.

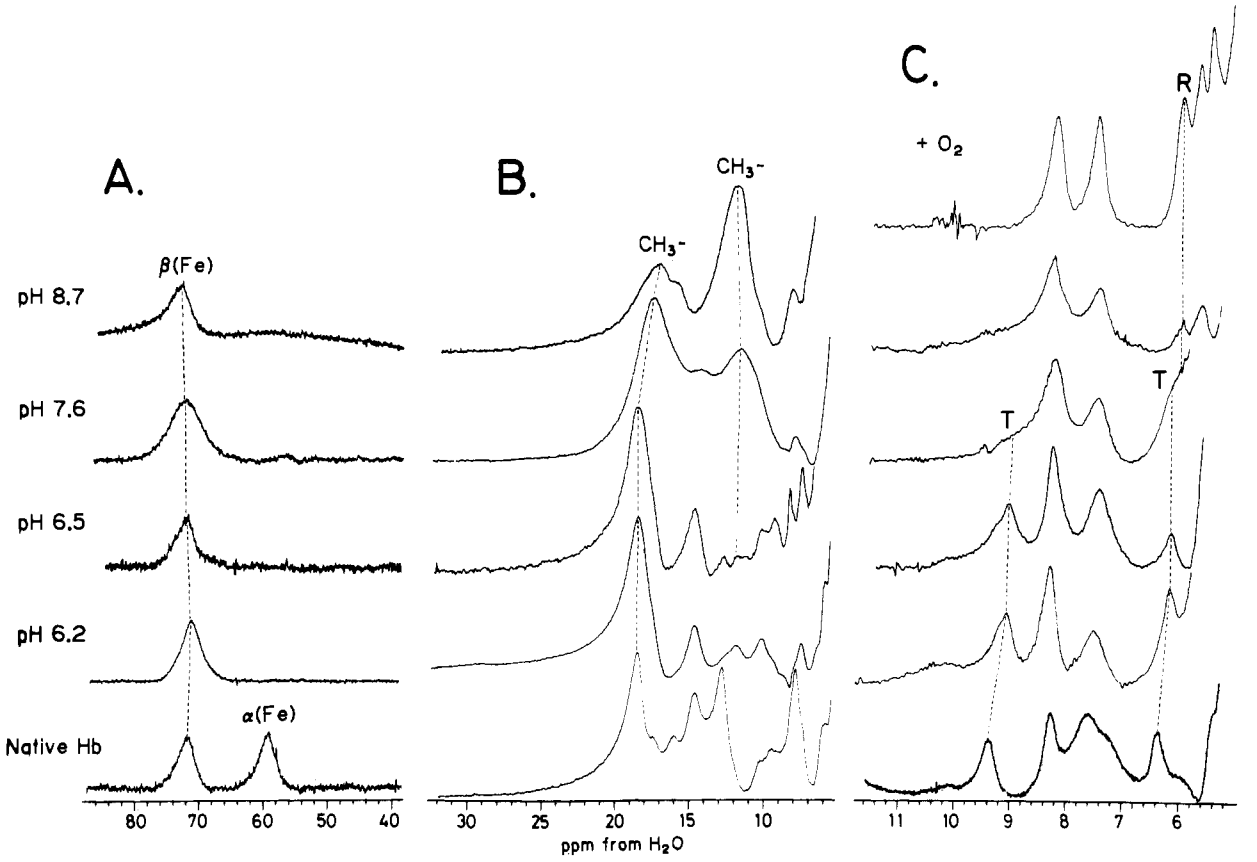

FIGURE 3: Proton NMR spectra (300 MHz) of deoxy native Hb (pH 7.0) (lowest spectrum), $\alpha(Ru-CO)_2\beta(Fe)_2$, and $\alpha(Ru-CO)_2\beta(Fe-O_2)_2$ (pH 7.0) (top spectrum in panel C) in 50 mM Bis-Tris or Tris buffer with 0.1 M chloride at 23 °C: hyperfine-shifted proton resonances of proximal His N_1H (A) and heme methyl group (B); hydrogen-bonded proton resonances (C). T and R show the T-state marker and R-state marker, respectively.

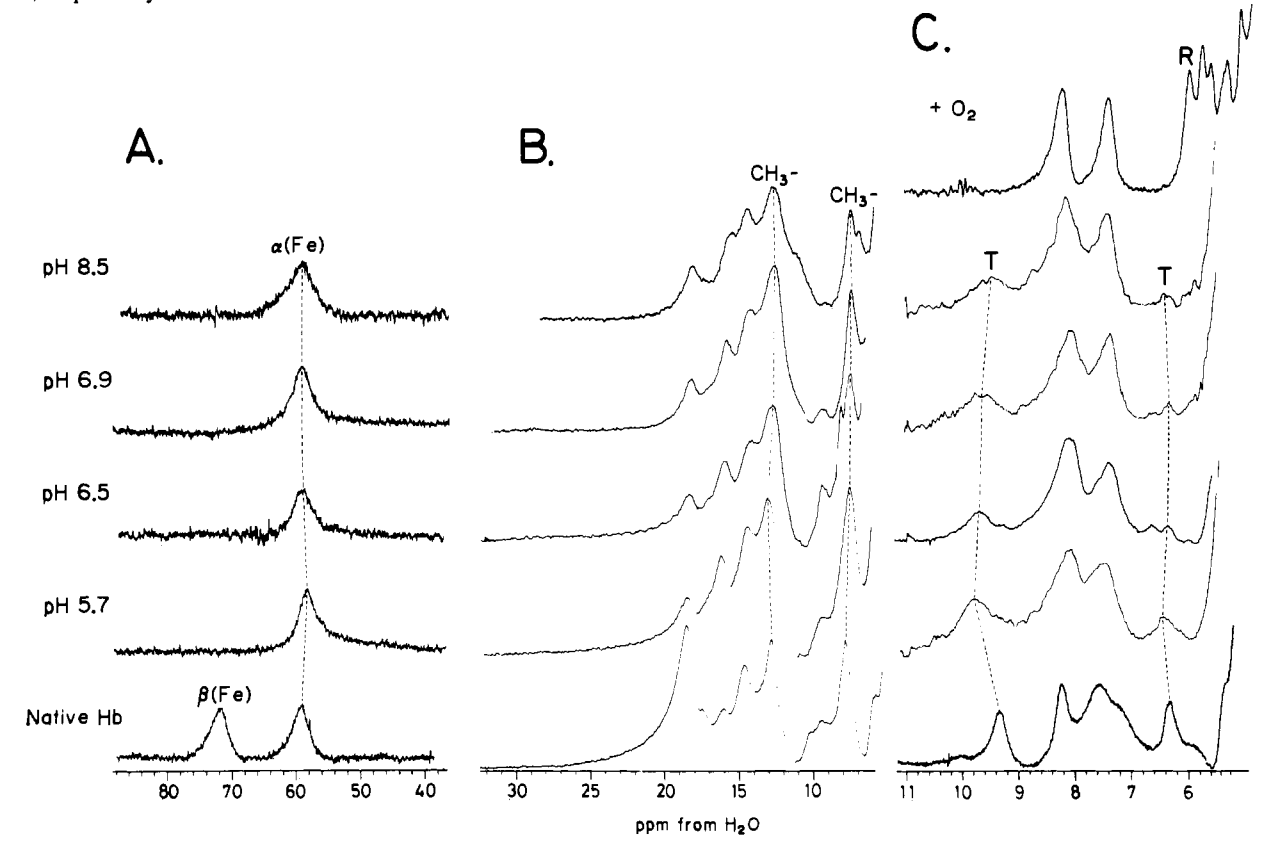

FIGURE 4: Proton NMR spectra (300 MHz) of deoxy native Hb (pH 7.0) (lowest spectrum), $\alpha(Fe)_2\beta(Ru-CO)_2$, and $\alpha(Fe-O_2)_2\beta(Ru-CO)_2$ (pH 7.0) (top spectrum in panel C) in 50 mM Bis-Tris or Tris buffer with 0.1 M chloride at 23 °C: hyperfine-shifted proton resonances of proximal His N_1H (A) and heme methyl group (B); hydrogen-bonded proton resonances (C). T and R show the T-state marker and R-state marker, respectively.

Table II: Resonance Positions of Native Hb, Ru-HbCO, and Ru–Fe Hybrid Hbs

Hb	proximal N_1H		heme methyl				hydrogen bond				
	β	α	β	α	β	α	T			T	R
native oxy Hb								8.2	7.4		5.9
$\alpha(Ru–CO)_2\beta(Ru–CO)_2$								8.2	7.4		6.0
$\alpha(Ru–CO)_2\beta(Fe–O_2)_2$								8.1	7.4		5.9
$\alpha(Ru–CO)_2\beta(Fe)_2$											
pH 8.7	72.9		17.0		11.8			8.2	7.4		5.9
pH 7.6	72.2		17.4		11.5			8.2	7.4		
pH 6.5	71.7		18.5				9.0	8.2	7.4	6.1	
pH 6.2	71.3		18.5				9.0	8.2	7.4	6.1	
$\alpha(Fe–O_2)_2\beta(Ru–CO)_2$								8.2	7.4		5.9
$\alpha(Fe)_2\beta(Ru–CO)_2$											
pH 8.5		58.7		12.8		7.8	9.5	8.2	7.4	6.4	
pH 6.9		58.7		12.8		7.7	9.6	8.1	7.4	6.4	
pH 6.5		58.7		13.0		7.5	9.7	8.1	7.4	6.4	
pH 5.7		58.6		13.0		7.6	9.8	8.1	7.5	6.4	
native deoxy Hb	72.1	59.5	18.3	12.6		7.6	9.4	8.3	7.6	6.4	

3C). The former signal is observed at 9.4 ppm for deoxy native Hb A and has been assigned to the $\alpha_1\beta_2$ intersubunit hydrogen bond between tyrosine-α42 (C7) and aspartic acid-β99 (G1) (Fung & Ho, 1975), and the latter at 6.3 ppm for native Hb A has been assigned to the intrasubunit hydrogen bond between valine-β98 (FG5) and tyrosine-β145 (HC2) (Viggiano et al., 1978). As the pH is raised, these T-marker signals decrease in intensity, and at pH 8.7, a small new resonance is detected at 5.9 ppm, which was also observed for the fully liganded hybrid, $\alpha(Ru–CO)_2\beta(Fe–O_2)_2$, and may be assigned to the "R-state" marker signal (Fung & Ho 1975).

Different features in the spectra of the intersubunit hydrogen-bonded protons are noticed for $\alpha(Fe)_2\beta(Ru–CO)_2$. The substitution of the native heme by Ru-DPIXCO in the β subunits reduced the intensity of the T-state marker substantially at 9.7 ppm but the signal was observed at pH 8.5 (Figure 4C). Another T-state marker, which is observed as a single peak at 6.4 ppm for deoxy native Hb (Figure 4C) and 6.1 ppm for the other hybrid (Figure 3C), experienced a similar tendency. The R-state marker at 5.9 ppm for the oxy spectrum (Figure 4C) is absent in the spectra of the half-liganded species at various pH values. All the resonance positions of the hydrogen-bonded protons are summarized in Table II.

Discussion

Ru-HbCO and Native HbCO. Inspection of Figure 2 and Table II shows that there is essentially no difference between native HbCO and Ru-HbCO in their ^1H NMR spectra (5–10 ppm) of intra- and intersubunit hydrogen-bonded protons. The ring current shifted resonance at −5 to −7 ppm is also similar between native HbCO and Ru-HbCO. These results indicate that the quaternary structure, which is manifested by hydrogen-bonded protons located in the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ subunit interfaces, and the tertiary structure, visualized by the Val E11 methyl resonances, are essentially identical in Ru-HbCO and native HbCO. Keeping in mind that the porphyrin substitution from protoporphyrin to deuteroporphyrin induces only localized conformational changes (Seybert & Moffat, 1976; Ishimori & Morishima, 1986), it is likely that the Ru-HbCO can serve as a model for the "fixed" oxy-like state and the Ru–Fe hybrid Hbs used here could work as the models for the partially liganded Hb.

Ligation Effect on the Tertiary and Quaternary Structure of Ru–Fe Hybrid Hbs. We have demonstrated that the heme substitution from native heme to Ru-DPIXCO for one subunit induces slight changes in the hydrogen-bonded and hyperfine-shifted proton resonances as shown in Table II. The T-state marker at approximately 9 ppm was observed for both hybrids (9.4 ppm for native Hb, 9.0 ppm for α-liganded hybrid, and 9.5–9.8 ppm for β-liganded hybrid). Another T-state marker around 6 ppm appeared in the β(Fe)-containing hybrid with decreased intensity in the complementary hybrid, whereas the R-state marker is only observed at high pH in $\alpha(Ru–CO)_2\beta(Fe)_2$. Since these T-marker signals are slightly shifted from those of native Hb A, we can conclude that the quaternary structures of the two hybrids at low pH are in the T-like state, which is defined as a quaternary structure somewhat different from the usual T state, as also found for the fully liganded valency hybrid in the presence of IHP (Morishima et al., 1986a,b), cross-linked valency hybrid Hbs (Miura & Ho, 1984), and asymmetric Fe–Co hybrid Hbs (Inubushi et al., 1986). The resonances of the proximal N_1H and the heme methyl group also appeared at almost the same position as those of native deoxy-Hb at low pH, and this is consistent with the features of the hydrogen-bonded proton resonances. This implies that the tertiary structure of the two hybrids remains in the deoxy-like structure.

It thus follows that the R state is not induced by the binding of two ligands at low and neutral pH. The crystal structure study of the half-liganded hybrid Hb, $\alpha(Fe–CO)_2\beta(Mn)_2$ (Arnone et al., 1986), also revealed that its quaternary and tertiary structure is almost identical with that of native deoxy-Hb. On the basis of MWC model analysis of the oxygen equilibrium curve, a swtich-over point, i_s, which expresses the degree of oxygenation at which the populations of the R and T state are equal, was determined to be in the range 2–3 (Imai, 1983), suggesting that the R–T transition is induced by the binding of the third oxygen. These results appear to be consistent with our present finding that the doubly liganded Hb is still in the T-like state. However, Simolo et al. (1985) reported that the $\alpha(Fe–CO)_2\beta(Zn)_2$ hybrid Hb exhibits the R-state marker signal. The R-state marker was also observed for iron–cobalt symmetric hybrid hemoglobins (Yonetani, personal communication). These results suggest that ligation of the two ligands induces quaternary structural transition from the T to the R state, which is contrary to our results. Such a difference may arise from the different state of the heme iron in the hybrid Hbs and/or the different properties of the substituted heme–metal. Our Ru–Fe hybrid Hbs have deoxygenated iron, whereas the heme iron in Co–Fe and Zn–Fe hybrid Hbs is liganded. These substituted metals also have different ionic radii, bond lengths to the axial ligands, and other properties. In other words, the difference in the quaternary structures of these half-liganded hybrid Hbs reflects these structural differences.

Difference of the Tertiary and Quaternary Structures of Two Complementary Ru–Fe Hybrid Hemoglobins. In order

to characterize the tertiary and quaternary structure of diliganded Ru–Fe hybrid Hbs, we examined the pH dependence of their NMR spectra.

In the hydrogen-bonded region of the ^1H NMR spectra of these two symmetric Fe–Ru hybrid Hbs, we demonstrated that heme substitution induces some different structural alterations between these two complementary hybrids. In the liganded α subunit hybrid $[\alpha(Ru–CO)_2\beta(Fe)_2]$ the T-state markers at approximately 6 and 9 ppm almost maintained their signal intensities at low pH. As the pH is raised, the T-state marker signals decreased their intensities, and a small R-state marker signal appeared as illustrated in Figure 3 and Table II, implying that the quaternary structure is converted from the T-like state into the R-like state at high pH. In the hyperfine-shifted spectral region, a new heme methyl resonance appeared concomitantly with the decrease of the signal intensity of the peak around 18 ppm, which has been assigned to the deoxygenated β(Fe) subunits. Since such a pH dependence of the heme methyl resonances is associated with the quaternary structural change, the pH-induced quaternary structural changes of the α(Ru–CO) hybrid are accompanied by the tertiary structural alterations in the heme environments.

These results may show that small free-energy difference between two quaternary structures (ΔG_2^{trans}) for the α-liganded hybrid is enough to change its quaternary structure by varying pH. Such a small free energy was also estimated on the basis of the oxygen equilibrium curve for native Hb A. Imai (1979) reported that ΔG_2^{trans} is approximately $+9 \times 10^3$ J at pH 6.5 and -3×10^3 J at pH 9.1, showing that the T-state is more favored at low pH. This appears to be consistent with our present results.

The complementary hybrid $[\alpha(Fe)_2\beta(Ru–CO)_2]$ exhibited a pH dependence which is quite different from that of α-$(Ru–CO)_2\beta(Fe)_2$. At low pH, two T-state markers, which are shifted slightly from those for native protein and exhibit the reduced intensities, are observed, showing that the β-liganded hybrid also maintains its quaternary structure in the T-like state. At pH 8.5 where the quaternary structure of the α-liganded hybrid is converted into the R-state, the spectrum of the β-liganded hybrid is essentially the same as that at low pH. The hyperfine-shifted resonances were also insensitive to varying pH as shown in Table II. This implies that the quaternary and teritary structure of the β-liganded hybrid is still in the T-like state at high pH, but not in equilibrium between R- and T-like states as found for the α-liganded hybrid. This observation also suggests that the free-energy difference (ΔG_2^{trans}) for the β-liganded hybrid is positive at high pH and its pH dependence is different from that of native Hb and the complementary hybrid. From the above discussion it is concluded that the β-liganded hybrid exhibits more T-state character than the α-liganded one at high pH, which suggests that the ligation for α subunits affects more substantially the quaternary and tertiary structures of the partner subunits than does the ligation for β subunits.

Such a different behavior between two complementary hybrids has also been encountered for several metal hybrid Hbs (Inubushi et al., 1983, 1986; Simolo et al., 1985; Shibayama et al., 1987). In Co–Fe hybrid Hbs (Inubushi et al., 1983, 1986), the ligation for the β subunits induced larger conformational changes in its quaternary structure, whereas Zn–Fe (Simolo et al., 1985) and Ni–Fe (Shibayama et al., 1987) hybrids exhibited preferential structural changes by the ligand binding of α subunits. $\alpha(Ru–CO)_2\beta(Fe)_2$ shows marked pH dependence for the hyrogen-bonded proton resonances, but $\alpha(Fe–CO)_2\beta(Ni)_2$ experiences no significant pH-dependent spectral changes in the same region, and the spectrum of the complementary hybrid $[\alpha(Ni)_2\beta(Fe–CO)_2]$ depends on pH as found for $\alpha(Ru–CO)_2\beta(Fe)_2$. Since many controversial points still remain open to further studies about the relationship between the partial ligand binding in one subunit and the quaternary structure, it is premature to conclude that there is an unequivocal correlation between ligand binding and the quaternary structure in the complementary pairs of hybrids. It may be safer to say that the quaternary structure of the half-liganded hybrids is not determined uniquely and is easily perturbed by the physical properties of the substituted metals or the specific structural changes of the subunits.

In summary, the present NMR results have revealed the structures of the half-liganded Fe–Ru hybrid Hbs which may serve as a model for the intermediate species in oxygenation of native Hb. The partial ligation of one subunit does not give rise to the drastic quaternary and heme environmental structural changes in the partner subunits at low pH. In other words, the half-liganded Ru–Fe hybrid Hbs maintain their quaternary structure in the T-like state, and the T to R structural transition can be induced by binding of three ligand molecules. However, the substantial structural changes accompanied by the quaternary structural transition which are induced at high pH for the α-ligand hybrid suggest that the energy difference between two quaternary structures of the α-liganded hybrid Hb is small. More detailed studies on the properties of the half-liganded Fe–Ru hybrid Hbs such as oxygen binding affinity are now under way.

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Thymic Humoral Factor γ2: Purification and Amino Acid Sequence of an Immunoregulatory Peptide from Calf Thymus

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ABSTRACT: Thymic humoral factor γ2 (THF-γ2), an octapeptide essential for immune regulation, was purified from calf thymus. The purification of THF-γ2, monitored in vitro and in vivo in mouse splenocyte proliferation assays, was achieved by gel filtration of low molecular weight thymus extracts followed by ion-exchange chromatography and sequential reversed-phase high-performance liquid chromatography. The process yielded 5 μg of THF-γ2/1000 kg of thymus tissue. The concentration of THF-γ2 required for augmentation of lymphocyte proliferation and interleukin 2 production was 5 ng/mL in vitro and 10 ng/kg per mouse in vivo. THF-γ2 has the amino acid sequence Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu. The proposed structure has been confirmed because a peptide was synthesized on the basis of this sequence that showed activity identical with that of the biological molecule. It shows no homology to the amino acid sequence of other thymic hormones nor is it part of any peptide or protein of known sequence. THF-γ2 retains essentially all of the biological activity of the thymus extract from which it is derived.

The immunological importance of the endocrine thymus is well established (Bach, 1976). The view that thymic hormones play a critical role in the regulation of immunocompetence developed from observations by us and others that implantation of thymus grafts in cell-impermeable diffusion chambers (Levey et al., 1963a,b; Osoba & Miller, 1963) or injection of thymus extracts (Small & Trainin, 1967; Hand et al., 1970; Trainin & Linker-Israeli, 1967) into neonatally thymectomized (NTx)[1] mice could reconstitute the resultant profound deficit in humoral and cell-mediated immunity. Previous research in our laboratories has demonstrated that in animal models, a crude extract of calf thymus denoted thymic humoral factor (THF) is essential for induction of clonal expansion, differentiation, and maturation of T-cell subsets (Kook & Trainin, 1974; Trainin et al., 1975, 1985). THF augmented most T-cell functions, such as the response to T-cell lectins, mixed lym-

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[1] Abbreviations: THF, thymic humoral factor; T-cell, thymus-derived lymphocytes; TFA, trifluoroacetic acid; IL-2, interleukin 2; Con A, concanavalin A; PHA, phytohemagglutinin; MLR, mixed lymphocyte reaction; NTx, neonatally thymectomized; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; FCS, fetal calf serum; Da, dalton(s); FTS, serum thymic factor; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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