NMR Study of Hybrid Hemoglobins Containing Unnatural Heme: Effect of Heme Modification on Their Tertiary and Quaternary Structures†

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ABSTRACT: The effect of heme modification on the tertiary and quaternary structures of hemoglobins was examined by utilizing the NMR spectra of the reconstituted [mesohemoglobin (mesoHb), deuterohemoglobin (deuteroHb)] and hybrid heme (meso–proto, deutero–proto) hemoglobins (Hbs). The heme peripheral modification resulted in the preferential downfield shift of the proximal histidine N_1H signal for the β subunit, indicating nonequivalence of the structural change induced by the heme modification in the α and β subunits of Hb. In the reconstituted and hybrid heme Hbs, the exchangeable proton resonances due to the *intra*- and *inter*subunit hydrogen bonds, which have been used as the oxy and deoxy quaternary structural probes, were shifted by 0.2–0.3 ppm from that of native Hb upon the β-heme substitution. This suggests that, in the fully deoxygenated form, the quaternary structure of the reconstituted Hbs is in an "imperfect" T state in which the hydrogen bonds located at the subunit interface are slightly distorted by the conformational change of the β subunit. Moreover, the two heme orientations are found in the α subunit of deuteroHb, but not in the β subunit of deuteroHb, and in both the α and β subunits of mesoHb. The tertiary and quaternary structural changes in the Hb molecule induced by the heme peripheral modification were also discussed in relation to their functional properties.

The cooperative phenomenon, commonly known as the "heme–heme" interaction in hemoglobin (Hb), has been the subject of intensive research during the past two decades. Despite considerable efforts devoted to the Hb molecule, the detailed molecular mechanism of the cooperative oxygenation of Hb is neither fully understood nor agreed upon by researchers. Comparing the X-ray crystallographic structures of human deoxyhemoglobin (deoxyHb) and oxyhemoglobin-like horse methemoglobin (metHb), Perutz (1970) proposed a stereochemical mechanism for the cooperative oxygenation of Hb, in which the correlation between the cooperativity and the R–T quaternary structural transition is emphasized. According to Perutz's model, the tertiary structural changes take place when a subunit is oxygenated, accompanied by a single concerted quaternary structural R–T transition which is responsible for the cooperativity of the oxygenation process. One of the unsolved problems in the R–T transition mechanism is how the structural change at the heme side is transmitted to the protein and why the changes of tertiary and quaternary structures are induced. Perutz (1970) has suggested the importance of the iron–ligand bond between the proximal histidine F8 imidazole and the heme in controlling the Hb oxygen affinity. Upon oxygenation, the iron atoms in the α and β hemes that are out of the heme plane in deoxyHb move into the center of the heme plane, thereby "triggering" the tertiary and eventually quaternary structural changes in the Hb molecule.

The heme maintains its relative position in the heme pocket by the coordination and the nonbonding interactions between the heme and the proximal histidine. The characteristic properties of Hb could be also determined by the nonbonding interactions such as van der Waals contacts between the heme and the protein, which amount to about 60 within 4 Å of the heme. Some substitutions of amino acid residues in the heme vicinity are known to induce serious functional defects. With the aim to disclose the role of the "nonbonding interactions" in the cooperative oxygenation, a number of investigations of the effect of chemical modification of the heme side chains on the structural and functional properties have been conducted for the reconstituted Hb. Antonini et al. (1959) reported that deuterohemoglobin (deuteroHb), which contains deuteroheme (2,4-H–) instead of protoheme (2,4-CH_2=CH—), has high oxygen affinity and low cooperativity and that mesohemoglobin (mesoHb) containing mesoheme (2,4-CH_3CH_2–) exhibits higher oxygen affinity and lower cooperativity than deuteroHb (Rossi-Fanelli et al., 1959). Recently, a more detailed study of the Hb derivatives reconstituted with the various synthetic hemes was presented by Kawabe et al. (1982). Further, Sugita et al. (1972, 1975; Makino & Sugita, 1976) examined functional properties of the Hb derivatives bearing hybrid hemes in the α and β subunits, $\alpha(meso)_2\beta(proto)_2$ and $\alpha(proto)_2\beta(meso)_2$. The most uncompromising problem that we encounter in studying the effect of nonbonding interactions on the cooperative phenomenon by utilizing the reconstituted Hbs is the uncertainty of their tertiary and quaternary structures. For example, visible spectra have been examined for the various reconstituted Hb derivatives, but their tertiary and quaternary structural features have not been delineated. The X-ray crystallographic studies for such reconstituted Hbs have not been made, due possibly to their unstability. In this sense, the NMR spectral studies are expected to contribute to the structural elucidation of such reconstituted Hbs.

In this study, we have tried to look at the effect of perturbation of the nonbonding interactions exerted by changing the heme peripheral group on the tertiary and quaternary structures of the Hbs by the use of high-resolution proton NMR for deuteroHb, mesoHb, and deutero–proto and meso–proto hybrid Hbs. Here we paid much attention to the exchangeable proton resonances due to the *inter*- and *intra*-subunit hydrogen bonds, hyperfine-shifted resonances for the proximal histidyl N_1H, and the ring-current-shifted E11 valine methyl signals as well. These resonances are now established

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to serve as the sensitive probes for tertiary and quaternary structural alterations. The results will be also discussed in relation to the cooperative oxygen affinities for these reconstituted Hbs.

Materials and Methods

Human adult Hb was prepared in the usual manner from fresh whole blood obtained from a normal individual. Stripped hemoglobin was obtained by gel filtration on Sephadex G-25 at pH 7.5 (Bermann et al., 1971). Apohemoglobin (apoHb) was prepared by the treatment of metHb with HCl/methyl ethyl ketone (MEK) (Yonetani, 1967) by modifying Teale's method (Teale, 1959). Crystalline mesohemin and deuterohemin were dissolved in a minimal volume of aqueous 50% pyridine solution before the recombination experiments. The hematin solution was added dropwise into a stirring solution of apoHb in 10 mM potassium phosphate buffer, pH 7.0. After addition of a small amount of dithionite, the excess hemin, dithionite, and pyridine were removed by passing through a column of Sephadex G-25 that had previously been equilibrated with 10 mM phosphate buffer, pH 6.5. The reconstituted Hb was purified on a CM-52 column from which the Hb was eluted with 50 mM potassium phosphate buffer, pH 7.0. All procedures were performed in a cold room (0–5 °C).

The oxygen equilibrium curves of the reconstituted Hbs were determined by the automatic recording method of Imai et al. (1970). The p_{50} and n_{max} values were identical with those previously available (Rossi-Fanelli & Antonini, 1959; Rossi-Fanelli et al., 1959; Kawabe et al., 1982).

The isolated α and β chains were prepared by using the method of Gerai et al. (1969). Hybrid Hbs were prepared as reported by Makino and Sugita (1978).

Proton NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Typically, an 11-μs (180°) radio frequency pulse and a ±36-kHz spectral width were applied to detect the proximal histidine N_1H signal and pyrrole proton for deuteroHb. A conventional water eliminated Fourier transform (WEFT) pulse sequence (180°–τ–90°–acquire) was used in order to minimize the water signal. A careful setting of the τ value (typically 90–100 ms) can completely eliminate the H_2O signal under rapid repetition of the sequence; regularly, an interpulse time of about 0.1 s was applied. We used the Redfield 2–1–4 pulse sequence with a 28–29-μs pulse and 8 K data points over a 6-kHz spectral width for obtaining proton resonance in the upfield region (ring-current-shifted resonances) and in the downfield region (exchangeable proton resonances in the subunits interface). The carrier frequency was in each case placed at 3 kHz upfield and 3.3 kHz downfield from the H_2O resonance. Proton shifts were referenced with respect to the water proton signal, which is 4.8 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 23 °C.

Results

Mesohemoglobin and Meso–Proto Hybrid Hemoglobins. In Figure 1 are shown the hyperfine-shifted proton resonances for native deoxyHb protohemoglobin (protoHb), mesoHb, and their hybrid derivatives. Figure 1D illustrates that deoxymesoHb in H_2O solution yields two distinct hyperfine-shifted proton signals in an extremely low-field region at 76.3 and 61.3 ppm. These two peaks were absent under the same pH and buffer conditions in D_2O solution. Similar large downfield paramagnetic shifts have been shown for native deoxyHb (Figure 1A) and assigned to the imidazole N_1H proton of the

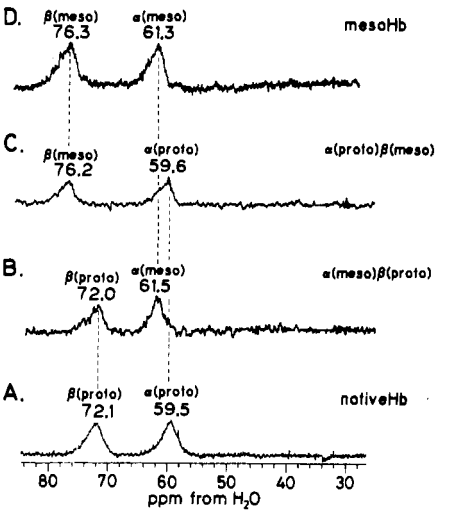

FIGURE 1: Comparison of the paramagnetic proton NMR (300-MHz) spectra between native Hb and mesoHb: (A) native deoxyHb; (B) deoxy $\alpha(meso)_2\beta(proto)_2$; (C) deoxy $\alpha(proto)_2\beta(meso)_2$; (D) deoxy mesoHb. All the samples were prepared in 50 mM Bis-Tris buffer at pH 6.5 and 23 °C.

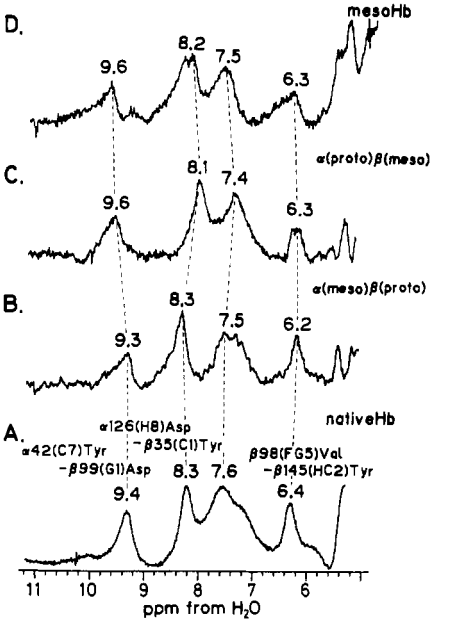

FIGURE 2: Comparison of the proton NMR (300-MHz) spectra between native Hb and mesoHb: (A) native deoxyHb; (B) deoxy $\alpha(meso)_2\beta(proto)_2$; (C) deoxy $\alpha(proto)_2\beta(meso)_2$; (D) deoxymesoHb. All the samples were prepared in 50 mM Bis-Tris buffer at pH 6.5 and 23 °C.

iron-bound proximal histidine F8 (La Mar et al., 1980): these two resonances at 72.1 and 59.5 ppm have been identified as the β and α subunits, respectively (Takahashi et al., 1980). Comparison of the proton NMR spectra between $\alpha(meso)_2\beta(proto)_2$ ($\alpha^M\beta^P$) (Figure 1C) and mesoHb (Figure 1D) shows that the signal at 76.3 ppm for mesoHb is shifted to 72.0 ppm for $\alpha^M\beta^P$, which is close to the N_1H signal position for the proto β subunits in native Hb. The signal at 61.5 ppm, which is seen in both mesoHb and $\alpha^M\beta^P$, can be readily assigned to the proximal histidine in the meso α subunit. Therefore, the remaining signal at 76.3 ppm for mesoHb is identified as the N_1H resonance of the meso β subunit. Similar assignment was made by comparing the NMR spectra between $\alpha(proto)_2\beta(meso)_2$ ($\alpha^P\beta^M$) (Figure 1B) and mesoHb (Figure 1D), as shown in the figure.

Figure 2 illustrates the exchangeable proton resonances for deoxymesoHb and meso–proto hybrid Hb. The peak at 9.4

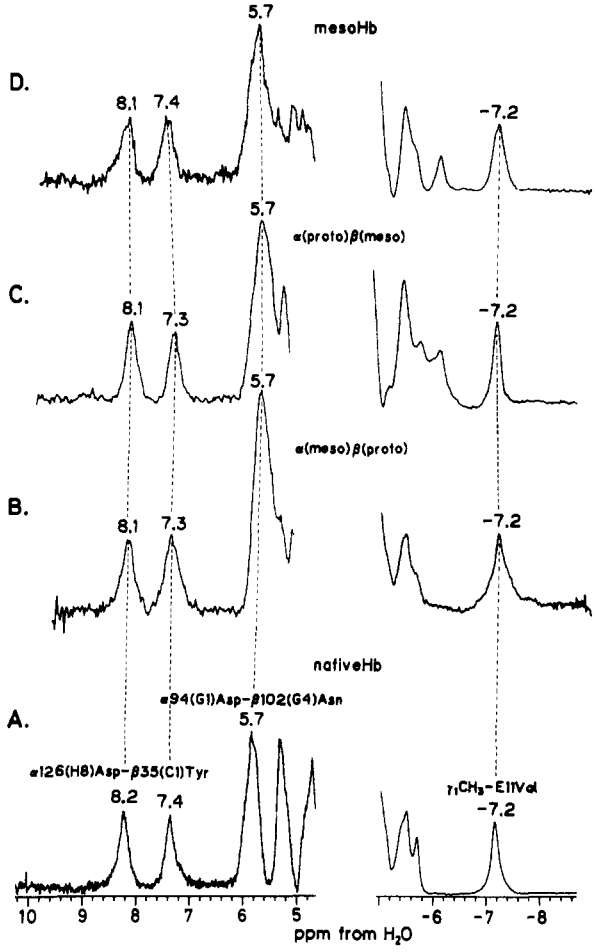

FIGURE 3: Comparison of the proton NMR (300-MHz) spectra between native Hb and mesoHb: (A) native oxyHb; (B) oxy $\alpha(\text{meso})_2\beta(\text{proto})_2$; (C) oxy $\alpha(\text{proto})_2\beta(\text{meso})_2$; (D) oxymesoHb. All the samples were prepared in 50 mM phosphate buffer at pH 7.0 and 23 °C.

ppm for native deoxyHb ($\alpha^P\beta^P$) (Figure 2A), which has been assigned to the *inter*subunit hydrogen bond between α42 (C7) tyrosine and β99 (G1) aspartic acid in the $\alpha_1\beta_2$ subunit interface and shown to serve as a deoxy quaternary state marker (T marker) (Fung & Ho, 1975), is almost unchanged in its signal position in going from $\alpha^P\beta^P$ to $\alpha^M\beta^P$ (Figure 2B), while it exhibits a 0.2 ppm downfield shift in $\alpha^P\beta^M$ (Figure 2C). A 6.4 ppm resonance (t marker) for native Hb, which is also characteristic of the deoxy tertiary structure and is assigned to the *intra*subunit hydrogen bond between β98 FG5 Val and β145 HC2 Tyr (Viggiano et al., 1978), is shifted by 0.1–0.2 ppm for the mesoHb and meso–proto hybrid Hb. A resonance at 8.3 ppm, which has been assigned to the *inter*subunit hydrogen bond between α126 (H8) aspartic acid and β35 (C1) tyrosine (Asakura et al., 1976), experiences an upfield shift upon substitution of the β heme, while it is unchanged for the α-heme substitution.

In Figure 3 are shown the NMR spectra of mesoHbO_2 and meso–proto hybrid HbO_2 in 50 mM phosphate buffer, pH 7.0. For native Hb, the ring-current-shifted proton peak at −7.2 ppm, which has been assigned (Lindstrom et al., 1972) to the γ_1-methyl resonance of α and β E11 Val, is not resolved. For mesoHb (Figure 3D) and their hybrid Hbs (Figure 3B,C), the corresponding signal was observed at the same position as the *native* Hb. In the downfield region, a resonance at 8.2 ppm, which was assigned to the proton associated with the hydrogen bond between β135 C1 Tyr and α129 F9 Asp (Asakura et al., 1976), remains almost unchanged upon the heme substitution.

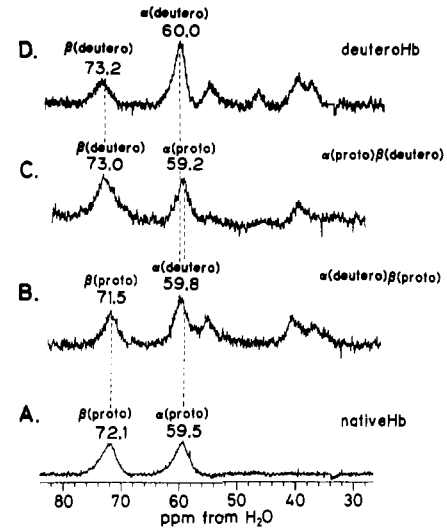

FIGURE 4: Comparison of the paramagnetic proton NMR (300-MHz) spectra between native Hb and deuteroHb: (A) native deoxyHb; (B) deoxy $\alpha(\text{deutero})_2\beta(\text{proto})_2$; (C) deoxy $\alpha(\text{proto})_2\beta(\text{deutero})_2$; (D) deoxydeuteroHb. All the samples were prepared in 50 mM Bis-Tris buffer at pH 6.5 and 23 °C.

A 5.9 ppm resonance, which has been utilized as an indicator for the oxy-like quaternary structure (Takahashi et al., 1980), is also insensitive to the heme substitution.

Deuterohemoglobin and Deutero–Proto Hybrid Hemoglobins. Figure 4 illustrates the hyperfine-shifted proton spectra of native deoxyHb, deuteroHb, and their hybrid Hb derivatives. For deoxydeuteroHb in H_2O (Figure 4D), several resonances appear in the 30–75 ppm region. The resonance at 73.2 ppm disappeared and the peak at 60.0 ppm lost its intensity in D_2O. By comparison of the spectra between $\alpha(\text{deutero})_2\beta(\text{proto})_2$ ($\alpha^D\beta^P$) (Figure 4C) and deuteroHb ($\alpha^D\beta^D$) (Figure 4D), the resonance at 73.2 ppm for deuteroHb in H_2O can be attributed to the N_1H proton of the proximal histidine of the β chain and the 60.0 ppm signal for deoxydeuteroHb to the α subunit.

The remaining signals between 55 and 35 ppm that are observed for both deuteroHb and deutero–proto hybrid Hbs are present even in D_2O. On the basis of the relative intensities with respect to the 73.2 ppm peak, these signals are not due to the ring methyl protons of the deuteroporphyrin. Therefore, the signals between 55 and 35 ppm and the resonance at 60.0 ppm superimposed on the N_1H signal may be assigned to the pyrrole β-protons at the 2,4-positions.

In the spectrum of deoxy-$\alpha^D\beta^P$, the signals at 54.9, 40.3, and 36.8 ppm may be due to the pyrrole protons at the 2,4-positions of the deuteroheme in the α subunit. Since the 59.8 ppm peak has a slightly larger intensity than the 72.1 ppm one, which has undoubtedly the single-proton intensity, the proximal N_1H and 2,4-H peaks for the deutero α subunit appear to be superimposed. It is therefore likely that four one-proton resonances consisting of two sets of 2,4-proton signals are observed in the 35–60 ppm region in Figure 4. Jue and La Mar (1984) showed that two sets of resonances found in the spectra of deoxydeuteroHb and deuteroHb$^+CN^-$ arise from the "native" and "disordered" forms with the heme rotated 180° about the α–γ–meso axis. However, it seems difficult to determine from the NMR spectra of deoxydeutero–proto hybrid Hb whether deuteroHb has two heme orientations or not. To disclose this problem, we examined the proton NMR spectra of the isolated chains of deoxydeuteroHb (Figure 5) and deuteroHb$^+N_3^-$ (Figure 6). As shown in Figure 5, the NMR spectra for the isolated chains of the deoxydeuteroHb

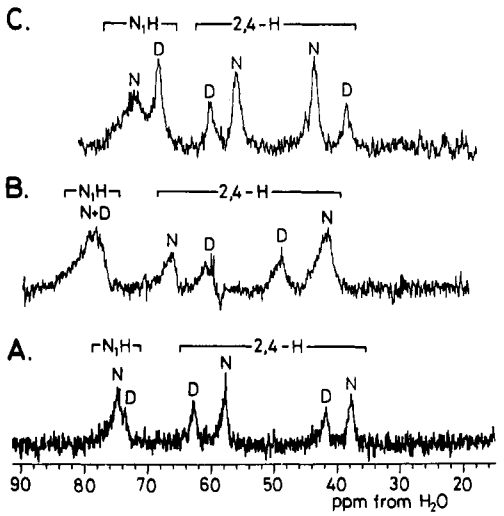

FIGURE 5: Downfield hyperfine-shifted portion of the NMR (300-MHz) spectra of (A) deuteroMb at 23 °C, (B) deuteroHb β chain at 4 °C, and (C) deuteroHb α chain at 23 °C in the deoxy complex form in 50 mM Bis-Tris at pH 6.5.

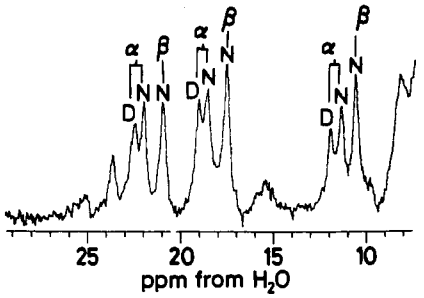

FIGURE 6: Downfield hyperfine-shifted portion of the NMR (300-MHz) spectrum of deuteroHb in the metazido complex form, in 50 mM Bis-Tris, pH 6.5, at 23 °C. N and D stand for the heme orientation in a native and disordered form, respectively. These assignments were made from the time course spectral change (see text).

exhibit almost the same spectra as those for deoxydeuteromyoglobin (deoxydeuteroMb) which has previously been shown to have the "heme disorder" (La Mar et al., 1978). This observation implies that the heme disorder is found in the isolated chains of deoxydeuteroHb as well as deoxydeuteroMb. However, in the spectrum of deuteroHb$^+N_3^-$ (Figure 6), the resonance from α subunit consists of two sets of signals, whereas β subunit exhibits only one. This may suggest that the orientation of the deuteroheme depends on whether it is embedded in Hb tetramer or in the isolated chains. For the tetrameric deuteroHb, the heme disorder exists only in the α subunit, not in the β subunit.

The NMR spectra (Figure 7) of the exchangeable proton resonances for deoxydeuteroHb and deutero–proto hybrid Hbs in 50 mM Bis-Tris[1] at 23 °C exhibit resonances at about 9.6, 8.3, 7.6, and 6.5 ppm. The substitution of the β heme in native Hb by the deuteroheme ($\alpha^P\beta^D$) (Figure 7C) results in a 0.2 ppm downfield shift of the signal at 9.4 ppm which is characteristic of deoxy quaternary structure in native Hb, while the α-heme substitution ($\alpha^D\beta^P$) (Figure 7B) experiences only 0.1 ppm upfield shift. A broad resonance at about 6.3 ppm in the spectrum of $\alpha^D\beta^D$ (Figure 7D) and well-resolved signals at about 6.3 ppm for $\alpha^D\beta^P$ and $\alpha^P\beta^D$ could be due to the hydrogen-bonded proton in the *intra*subunit of the β chain, by comparing the results for the native Hb (Viggiano et al., 1978). This signal is the t state marker which serves as a probe for the deoxygenated tertiary structure.

In the NMR spectra of oxygenated deuteroHb and deutero–proto hybrid Hbs, the exchangeable proton resonances appear at about 8.2 and 7.4 ppm, as shown in Figure 8. The resonance pattern in the 5–10 ppm region in Figure 8 is essentially the same as that for native Hb. Thus, the resonances in this region can be assigned to the *inter*subunit hydrogen bonds for deuteroHb and deutero–proto hybrid Hbs.

The Val E11 γ_1-methyl protons for deuteroHb exhibit the slightly resolved peaks at −7.2 ppm. This signal splitting may result from the nonequivalence of the heme environment of the α and β subunits, as observed for native HbCO in the presence of IHP (Lindstrom et al., 1972). If this signal splitting is due to two heme orientations in the α subunit as stated above, the signal pattern should be much more complicated by the heme disorder for the α subunit and by the α,β nonequivalence. As Figure 8 shows, the resolved two signals exhibit the same intensities, which was more clearly recognized upon the addition of IHP to deuteroHbCO (Morishima and Ishimori, unpublished data). It therefore follows that the Val E11 γ_1–methyl signal in deuteroHb does

Table I: Resonance Positions of the F8 Histidine N_1H for Reconstituted Hemoglobin and Their Assignments

	resonance position (ppm)	
	α subunit	β subunit
mesoHb ($\alpha^M\beta^M$)	61.3	76.3
$\alpha^M\beta^P$	61.5	72.0
$\alpha^P\beta^M$	59.6	76.2
deuteroHb ($\alpha^D\beta^D$)	60.0	73.2
$\alpha^D\beta^P$	59.8	71.5
$\alpha^P\beta^D$	59.2	73.0
protoHb (native Hb)	59.5	72.1

Table II: Resonance Positions of the Exchangeable Proton of Hydrogen Bonds for Deoxygenated Reconstituted Hemoglobin and Their Assignments

	resonance position (ppm)			
mesoHb	9.6	8.2	7.5	6.3
$\alpha^M\beta^P$	9.3	8.3	7.5	6.2
$\alpha^P\beta^M$	9.6	8.1	7.4	6.3
deuteroHb	9.6	8.2	7.5	6.6, 6.3
$\alpha^D\beta^P$	9.5	8.3	7.6	6.4, 6.1
$\alpha^P\beta^D$	9.6	8.2	7.4	6.6, 6.4
protoHb	9.4	8.3	7.6	6.4
assignment	α42 C7 Tyr– β99 G1 Asp	α126 H8 Asp– β35 C1 Tyr		β98 FG5 Val– β145 HC2 Tyr

Table III: Resonance Positions of the Exchangeable Proton of Hydrogen Bonds for Oxygenated Reconstituted Hemoglobin and Their Assignments

	resonance position (ppm)			
mesoHb	8.1	7.4	5.7	−7.2
$\alpha^M\beta^P$	8.1	7.3	5.6	−7.2
$\alpha^P\beta^M$	8.1	7.3	5.7	−7.2
deuteroHb	8.1	7.4	5.8	−7.1, −7.2
$\alpha^D\beta^P$	8.2	7.4	5.7	−7.2
$\alpha^P\beta^D$	8.3	7.5	5.8	−7.2
protoHb	8.2	7.4	5.8	−7.2
assignment	α126 H8 Asp– β35 C1 Tyr		α94 G1 Asp– β102 HC2 Tyr	E11 Val

[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; IHP, inositol hexakis(phosphate).

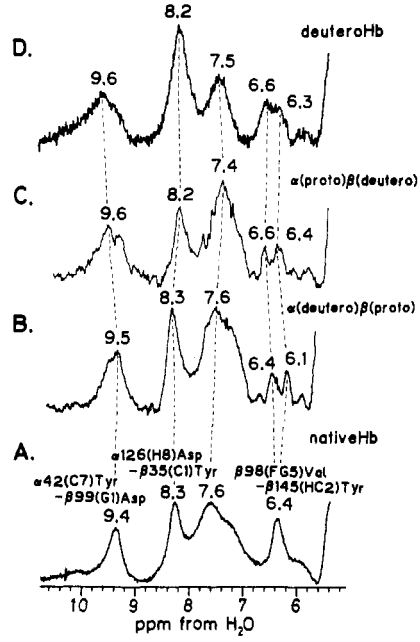

FIGURE 7: Comparison of the proton NMR (300-MHz) spectra between native Hb and deuteroHb: (A) native deoxyHb; (B) deoxy $\alpha(\text{deutero})_2\beta(\text{proto})_2$; (C) deoxy $\alpha(\text{proto})_2\beta(\text{deutero})_2$; (D) deoxydeuteroHb. All the samples were prepared in 50 mM Bis-Tris buffer at pH 6.5 and 23 °C.

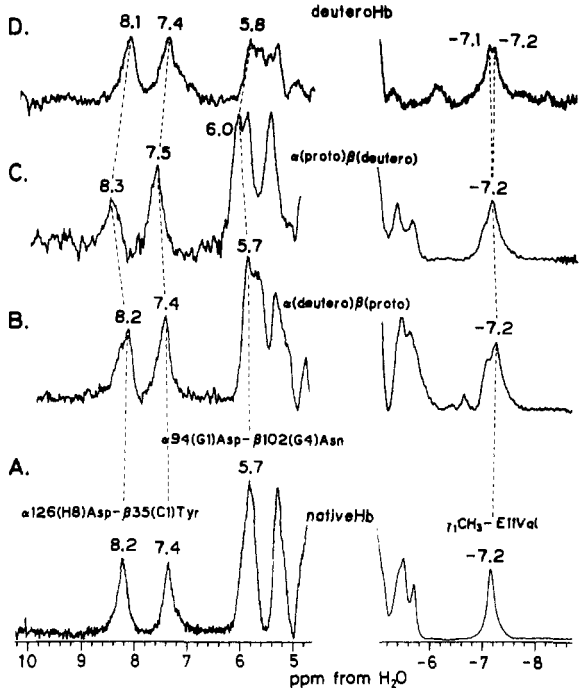

FIGURE 8: Comparison of the proton NMR (300-MHz) spectra between native Hb and deuteroHb: (A) native oxyHb; (B) oxy $\alpha(\text{deutero})_2\beta(\text{proto})_2$; (C) deoxy $\alpha(\text{proto})_2\beta(\text{deutero})_2$; (D) deoxydeuteroHb. All the samples were prepared in 50 mM phosphate buffer at pH 7.0 and 23 °C.

not sense the heme heterogeneity in the α subunit but rather responds to the α,β nonequivalence.

All the signal assignments and resonance positions are assembled in Tables I–III.

Discussion

From the present results of the proton NMR spectra of the reconstituted Hbs containing the unnatural heme, it is revealed that the modification of the heme side chains affects the tertiary and quaternary structure of the Hbs.

Tertiary Structure of Reconstituted Hemoglobins. In the deoxy form, the most outstanding feature of the effect of the heme substitution on the proximal histidyl N_1H proton signal in the reconstituted Hbs (Figure 1, Table I) is the preferential downfield shift for the deoxy meso β subunit compared with that for the meso α subunit. This is clearly seen in Figure 1 where the spectrum of the native deoxyHb is compared with that of mesoHb. This feature is also found for the hybrid Hbs. As figure 4 shows, deuteroheme-substituted Hbs also experience a similar trend. It thus follows that the modification of the heme side chains in the β subunits exerts a more subtle structural perturbation at the heme proximal side than does the heme side modification in the α subunit. It is also to be noted that the heme substitution in the α and/or β subunit does not appear to affect the heme proximal structure of the complementary subunit. This nonequivalence in the proximal structural changes induced by the heme substitution between the α and β subunits has also been encountered for the Co-substituted Hb and its Co–Fe hybrid Hb derivatives (Inubushi et al., 1983). A substantial difference in the van der Waals contacts in the heme environments between the α and β subunits could be responsible for the present finding.

In the oxygenated form, the Val E11 γ_1–methyl proton signal, which serves as a probe for the tertiary structure in the heme distal side, exhibits no significant difference between mesoHb, deuteroHb, native Hb, and their hybrid Hbs except the one for deuteroHb, which affords slightly resolved peaks at around −7.2 ppm due to the α,β nonequivalence. This may suggest that the tertiary structure of the oxygenated Hbs is more spacious in the heme vicinity than that of the deoxygenated form, so that the heme substituent hardly exerts the structural perturbation in the heme distal side for the oxygenated Hb.

It is also to be noted that the displacement of the proximal histidine N_1H shift in going from the native Hb to mesoHb is substantially larger than that in going from native Hb to deuteroHb. This is somewhat surprising in that a minor chemical modification of the heme vinyl substituents to ethyl groups produces more stereochemical perturbation in the heme proximal side than does the more drastic structural modification at the 2,4-positions for deuteroHb. However, these structural changes seem interesting in relation to the difference in the functional behavior of the ferrous forms of these two reconstituted Hbs (Rossi-Fanelli & Antonini, 1959; Rossi-Fanelli et al., 1959). MesoHb exhibits pronounced differences in the oxygen-binding properties from native Hb, while deuteroHb shows an oxygen affinity quite similar to that of native Hb. The X-ray structural study of horse metHb reconstituted with deutero- and mesoheme by Seybert and Moffat (1976, 1977) showed that numerous small structural changes in the heme environments are seen for horse metmesoHb, while horse deuteroHb experiences only minor and highly localized structural perturbations.

It is quite interesting to find in the present study that the heme disorder exists only in the α subunit and a unique heme orientation is maintained in the β subunit for deuteroHb.[2] However, from the X-ray crystallography of horse aquometdeuteroHb (Seybert & Moffat, 1976), the heme disorder is

[2] La Mar et al. (1985) have recently reported that nativeHbN_3^- experiences ~10% disorder in the β subunit and a single orientation dominates (estimated >98%) in the α subunit. The apparent difference in the equilibrium heme disorder between deuteroHb and native Hb could be caused by the change in the steric constraints of the heme periphery, in going from the native to disordered form.

not found, and deuteroheme combines with the globin with a unique native orientation in both α and β subunits. This difference between the results of the X-ray crystallography and that of the present NMR spectra suggests that the conversion from the disordered orientation to the native orientation could occur with lapse of time or by crystallization of deuteroHb. In fact, the time course spectra of deuteroHbN_3^- showed that the resonances arising from the disordered form lose their intensities in time and concomitantly the signals from the native orientation form increased (Morishima and Ishimori, unpublished data). Therefore, one should take into account the effect of the heme disorder on the functional properties of deuteroHb. Livingston et al. (1984) reported that the Mb sample containing the disordered heme orientation exhibits a higher oxygen affinity. On the other hand, the heme disorder is not found for mesoHb, although the heme heterogeneity has been found for mesoMb. It therefore follows that the protein structure of the Hb β subunit is more strictly constructed to accommodate the heme in the native orientation than that of Hb α subunit and that Mb is less strict than Hb for the steric recognition of the heme.

Quaternary Structure of Reconstituted Hemoglobins. As is shown in the NMR spectra of the *inter*subunit exchangeable proton resonances (Figures 2, 3, 7, and 8), the quaternary structures of the reconstituted Hbs containing the unnatural heme seem similar to those of native Hb. The resonances at 9.4 and 6.3 ppm in native deoxyHb have been shown to serve as the T and t marker signals (Fung & Ho, 1975). These signals were also observed in mesoHb, deuteroHb, and their hybrid Hbs, although their resonance positions were slightly shifted by 0.2–0.3 ppm from that of native Hb. This suggests that the heme peripheral modification induces quaternary structural changes in deoxyHb; that is, these hydrogen bonds are slightly distorted upon the heme substitution. Seybert and Moffat also pointed out on the basis of X-ray crystallography (Seybert & Moffat, 1976, 1977) that, in the met form of horse mesoHb and deuteroHb, the phenolic side chain of α42 C7 Tyr moves and some other structural changes occur at the first four residues of the G helix. It has been suggested that the T marker signal at 9.4 ppm in native Hb arises from the hydrogen bond between α42 C7 Tyr and β99 G1 Asp that is located at the α_1–β_2 *inter*subunit and this hydrogen bond plays an important role in the cooperative oxygenation. Some mutant Hbs that lack this hydrogen bond, such as Hb Yakima (β99 G1 Asp → His) (Novy, 1967), Hb Kempsey (β99 G1 Asp → Asn) (Reed, 1968; Perutz et al., 1974), and Hb Radcliffe (β99 G1 Asp → Ala) (Weatherall, 1977), cannot take a T state and exhibit the low cooperativity ($n \sim 1.0$). The distortion of the hydrogen bond interferes to form the T state, which has low oxygen affinity like native deoxyHb. Therefore, it is likely that the deoxy form of the reconstituted Hbs is in an intermediate form between the R and T states in native Hb, and it can be concluded that the quaternary structures of the reconstituted Hbs are in an "imperfect" T state even in the fully deoxygenated form.

As shown in Figures 3 and 8, one can find that the difference of the resonance pattern between reconstituted and native Hbs for the oxygenated form is smaller than that for the deoxygenated form. The resonance at 5.7 ppm, which is the R state marker (Fung & Ho, 1975), was also observed for reconstituted Hbs. Moreover, the resonances at 8.2 and 7.4 ppm observed for native Hb are little affected by the heme substitution. These findings imply that the quaternary structure of the reconstituted Hbs in the oxygenated form is more closely similar to that of the native Hb. Consequently, the structural

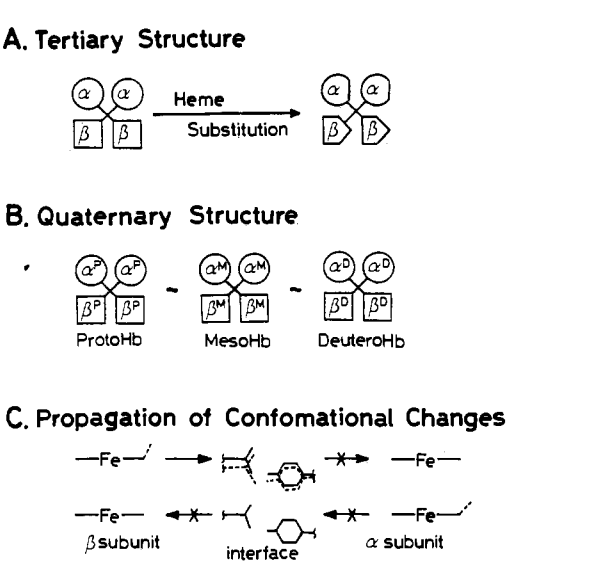

FIGURE 9: Schematic representations of the present results of the reconstituted Hbs. (A) The heme modification induces the quaternary structural change more effectively in the β subunit than in the α subunit. (B) The quaternary structures of the reconstituted Hbs are closely similar to that of the native Hb, although their deoxy quaternary structures are in an imperfect T state. (C) The heme environmental structural change induced by the modification of the β-heme peripheral substituent propagates to the subunit interface.

differences between native and reconstituted Hbs are more distinct in the deoxy state than in the oxy state. We thus conclude that low cooperativity in oxygenation for mesoHb and deuteroHb arises mainly from the distortion of the hydrogen bond in the α_1–β_2 subunit interface, which causes an imperfect T state for deoxyHb. The similar feature has been found in the globin-modified Hbs such as des-Arg-Hb, des-Arg-Tyr-Hb, and *N*-ethylsuccinimide–Hb that have low cooperativity and afford the T marker signals with a small shift (0.1–0.2 ppm) (Miura & Ho, 1984).

It is also of interest to note that in the deoxygenated form the resonance pattern around 9.4 and 8.3 ppm for $\alpha^P\beta^M$ is similar to that for $\alpha^M\beta^M$ and the one for $\alpha^M\beta^P$ is rather similar to that for $\alpha^P\beta^P$. The same features are also noticed for deutero–proto hybrid Hbs. It is then likely that the quaternary structure of tetrameric deoxyHb is affected by the structure of the β subunit more effectively than by the α subunit. This phenomenon is also reinterpreted as follows: the tertiary structural change induced by the heme peripheral modification is larger in the β subunit than in the α subunit as shown above. Therefore, the structural change in the heme environment of the β subunit is effective enough to slightly distort the hydrogen bond in the *inter*subunit region, whereas that in the α subunit is less effective. It thus follows that the structural change in the heme vicinity induced by the modification of the β heme can propagate to the *inter*subunit region, thereby affecting the quaternary structure of the reconstituted Hbs, and such structural change does not occur for the α subunit. As to the functional properties of the hybrid heme Hbs, Sugita et al. (1972) reported the Hill constants (n) for native Hb ($\alpha^P\beta^P$) ($n = 3.0$), $\alpha^M\beta^P$ ($n = 2.6$), $\alpha^P\beta^M$ ($n = 1.8$), and mesoHb ($\alpha^M\beta^M$) ($n = 1.6$). Cooperativity is more effectively influenced by the heme substitution of the β subunit than by that of the α subunit. This appears to parallel the present results from the NMR studies of the quaternary structures of the hybrid Hbs and to show that the small difference in the quaternary structure substantially affects the functional properties.

In summary, the perturbation of nonbonding interactions between the heme and the globin induces substantial changes

of both tertiary and quaternary structures (Figure 9). As to the tertiary structure, the iron–histidine bond is affected in deoxyHb although in the oxy form no significant structural changes are found by the heme peripheral modification. It has also become clear that the tertiary structural change is different between the α and β subunit in such a way that the β subunit is more readily affected by such a perturbation (Figure 9A) and that such structural change in the deoxy β subunit propagates only to the *inter*subunit region and induces slight distortion of the hydrogen bonds (Figure 9C). The quaternary structure of the reconstituted Hbs in the deoxygenated form is in an imperfect T state, which is slightly different from the T state of native Hb. This quaternary structural perturbation arising mainly from the β subunit exerts substantially large effects on the functional properties such as oxygenation cooperativity. Moreover, the heme disorder is confirmed only in the α subunit of deuteroHb. More detailed studies on the heme disorder in various Hb derivatives are now under way and will be reported in a forthcoming paper.

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REFERENCES

Asakura, T., Adachi, K., Wiley, J. S., Fung, L. W.-M., Ho, C., Kilmartin, J. V., & Perutz, M. F. (1976) *J. Mol. Biol. 104*, 185–195.

Bermann, M., Benesch, R., & Benesch, R. E. (1971) *Arch. Biochem. Biophys. 145*, 236–239.

Fung, L. W.-M., & Ho, C. (1975) *Biochemistry 14*, 2526–2535.

Gerai, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4664–4667.

Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., & Kuroda, M. (1970) *Biochim. Biophys. Acta 200*, 189–196.

Inubushi, T., Ikeda-Saito, M., & Yonetani, T. (1983) *Biochemistry 22*, 2904–2907.

Jue, T., & La Mar, G. N. (1984) *Biochem. Biophys. Res. Commun. 119*, 640–645.

Kawabe, K., Imaizumi, K., Yoshida, Z.-I., Imai, K., & Tyuma, I. (1982) *J. Biochem.* (Tokyo) *92*, 1713–1722.

La Mar, G. N., Budd, D. L., Viscio, D. B., Smith, K. M., & Langry, K. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5755–5759.

La Mar, G. N., Nagai, K., Jue, T., Budd, D. L., Gersonde, K., Sick, H., Kagimoto, T., Hayashi, A., & Taketa, F. (1980) *Biochem. Biophys. Res. Commun. 96*, 1172–1177.

La Mar, G. N., Yamamoto, Y., Jue, T., Smith, K. M., & Pandey, R. K. (1985) *Biochemistry 24*, 3826–3831.

Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehmann, H., & Ho, C. (1972) *Biochemistry 11*, 1677–1681.

Livingston, D. J., Davis, N. L., La Mar, G. N., & Brown, W. D. (1984) *J. Am. Chem. Soc. 106*, 3025–3026.

Makino, N., & Sugita, Y. (1978) *J. Biol. Chem. 253*, 1174–1178.

Miura, S., & Ho, C. (1984) *Biochemistry 23*, 2492–2499.

Morishima, I., & Hara, M. (1983) *J. Biol. Chem. 258*, 14428–14432.

Novy, M. J. (1967) *J. Clin. Invest. 46*, 1848–1852.

Perutz, M. F. (1970) *Nature (London) 228*, 726–739.

Perutz, M. F., Lander, J. E., Simon, S. R., & Ho, C. (1974) *Biochemistry 13*, 2163–2173.

Reed, C. S. (1968) *Blood 31*, 623–630.

Rossi-Fanelli, A., & Antonini, E. (1959) *Arch. Biochem. Biophys. 80*, 308–317.

Rossi-Fanelli, A., Antonini, E., & Caputo, A. (1959) *Arch. Biochem. Biophys. 85*, 37–42.

Seybert, D. W., & Moffat, K. (1976) *J. Mol. Biol. 106*, 895–902.

Seybert, D. W., & Moffat, K. (1977) *J. Mol. Biol. 113*, 419–430.

Sugita, Y. (1975) *J. Biol. Chem. 250*, 1251–1256.

Sugita, Y., Bannai, S., Yoneyama, Y., & Nakamura, T. (1972) *J. Biol. Chem. 247*, 6092–6095.

Takahashi, S., Lin, A. K.-A. C., & Ho, C. (1980) *Biochemistry 19*, 5196–5202.

Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.

Viggiano, G., Wiechelman, K. J., Chervenich, P. A., & Ho, C. (1978) *Biochemistry 17*, 795–799.

Weatherall, D. J. (1977) *Br. J. Haematol. 35*, 177–185.

Yonetani, T. (1967) *J. Biol. Chem. 242*, 5008–5013.