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Study of the Specific Heme Orientation in Reconstituted Hemoglobins†

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ABSTRACT: NMR studies of the recombination reaction of apohemoglobin derivatives with natural and unnatural hemes and of the heme-exchange reaction for reconstituted hemoglobin have revealed that the heme is incorporated into the apoprotein with stereospecific heme orientations dependent upon the heme peripheral 2,4-substituents and the axial iron ligand(s). Heme orientations also depend on whether recombination occurs at the α or β subunit and on whether or not the complementary subunit is occupied by the heme. In the recombination reaction with the azido complex of deuterohemin, the α subunit of the apohemoglobin preferentially combines with the hemin in the "disordered" heme orientation, whereas protohemin is inserted in either of two heme orientations. Mesohemin inserts predominantly in the "native" heme orientation. For the β subunit, specific heme orientation was also encountered, but the specificity was somewhat different from that of the α subunit. It was also shown that the specific heme orientation in both subunits is substantially affected by the axial heme ligands. These findings imply that apohemoglobin senses the steric bulkiness of both the porphyrin 2,4-substituents and the axial iron ligands in the heme–apoprotein recombination reaction. To gain an insight into the effect of the protein structure, the heme reconstitution reaction of semihemoglobin, demonstrating that the heme orientation in the reconstituted semihemoglobin with the azido–deuterohemin complex was in the native form, was also examined. Moreover, when protoheme is added to a solution of the deuteroheme-reconstituted hemoglobin so that the heme-exchange reaction takes place, the resulting heme orientation for the α subunit is also quite specific (native heme orientation only), which is different from the case of the heme–apoprotein recombination reaction. Therefore, it is concluded that the orientation of the heme inserted into the apoprotein of the hemoglobin subunit depends upon the structure of the complementary subunit. In other words, the subunit structural changes induced by the tertiary structural changes of the complementary subunit through the subunit–subunit interaction subtly affect the stereospecific heme orientation.

The apparently simple and instantaneous (~1 ms) reconstitution reaction in vitro of heme and apohemoglobin (apoHb)[1] to yield unique holoproteins (Gibson & Antonini, 1960; rose & Olson, 1983) leads to the view that the last step of the hemoprotein biosynthesis is a rapid folding process which affords the same species as found in single crystals (Gibson & Antonini, 1960; Adams, 1976, 1977). Recent ^{1}H NMR studies have demonstrated that the reconstitution of heme with apoMb and apoHb proceeds via a random isomeric incorporation, with respect to rotation about the α,γ-meso axis of

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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb A, human adult hemoglobin; metHb, ferric hemoglobin; Mb, myoglobin; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; ppm, parts per million; MEK, methyl ethyl ketone; WEFT, water-eliminated Fourier transform; semiHb, semihemoglobin; apoHb, apohemoglobin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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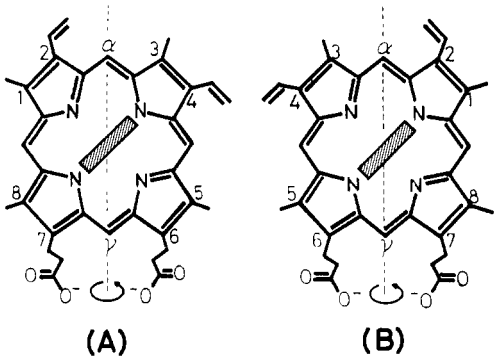

FIGURE 1: Two possible orientations of the heme relative to the proximal histidyl imidazole plane: (A) Form determined by X-ray crystallography of Hb A (Fermi, 1975); (B) heme rotated 180° about the α,γ-meso axis of form A. The shaded rectangel indicates the orientation of the proximal histidylimidazole plane.

heme, the result being defined as either the "native" or the "disordered" heme orientation (Figure 1) (La Mar et al., 1985; Yamamoto & La Mar, 1986). The disordered heme orientation goes to the native heme orientation within several hours to many days, but a small amount of the disordered isomer is present at equilibrium (La Mar et al., 1980, 1983, 1984, 1985; Jue et al., 1983).

We have recently found (Ishimori & Morishima, 1986) that the deuterohemin incorporated into apoHb is disordered only in the α subunit, with the native orientation present in the β subunit. This suggests that interactions between globin and heme could control the heme orientation in each subunit. To gain an insight into how these interactions control the specific heme orientations and to explain the mechanism for stereospecific heme insertion, we have performed ^{1}H NMR studies of the reconstitution reactions of some apoHb derivatives with a variety of hemin complexes bearing different heme substituents and axial heme ligands.

^{1}H NMR has been shown to be a powerful tool for the investigation of the heme orientation in hemoproteins (La Mar et al., 1985; Yamamoto & La Mar, 1986). The ^{1}H NMR measurements of the hemoproteins were made here as soon as possible after addition of the hemin complex to the apoHb solution to get the ratio of the native to disordered heme orientations. The results show that apoHb can stereospecifically recognize the heme orientations by the interactions between heme and globin at the time of recombination and that such a stereospecific recognition in one subunit depends upon the structure of the complementary subunit. It is concluded that the heme orientation is determined by the steric hindrance exerted by the heme peripheral groups and the axial ligand as well as by the tertiary structure of the complementary subunit.

Experimental Procedures

Sample Preparation. Human adult Hb was prepared in the usual manner from fresh whole blood obtained from a normal individual. Protohemin was obtained from Sigma Chemical Co. (bovine, type I) and used without further purification. Stripped Hb was obtained by gel filtration with Sephadex G-25 equilibrated with 0.01 M Tris-HCl buffer containing 0.1 M NaCl at pH 7.5 (Bermann et al., 1971). Apohemoglobin was prepared by the treatment of metHb with HCl/MEK (Yonetani, 1967) by modifying Teale's method (Teale, 1959; Ishimori & Morishima, 1986). Reconstituted Hbs were prepared as descrribed in the previous paper (Ishimori & Morishima, 1986). Crystalline deuterohemin, protohemin, and mesohemin were dissolved in a minimal volume of 0.1 N NaOD or 50% aqueous solution of pyridine before the recombination experiments. The hematin solution was diluted with D_2O containing the ligand and was added dropwise into a stirring solution of apoHb in 10 mM potassium phosphate buffer, pH 7.0. The reconstitutions with heme–CO complex were performed as reported by La Mar et al. (1985). Reconstituted Mbs (sperm whale) were prepared in a manner similar to that for reconstituted Hbs. After the buffer exchange to 50 mM Bis-Tris, pH 7.0, by ultrafiltration, the reconstituted protein was concentrated to about 1 mM. All the procedures were performed in a cold room (0–5 °C). SemiHbs were prepared according to the method of Cassoly and Banerjee (1971) and reconstituted with hemin in a manner similar to that for apoHb. For the heme-exchange reactions, the appropriate reconstituted holoprotein was diluted to $\sim$1 mM in D_2O containing 50 mM Bis-Tris, and then a 2 M equivalent of protohemin–N_3^- solution in 0.01 N NaOD was added.

For NMR measurements, all of the Hb samples, except for the metcyano complex, were converted to the metazido complex because this complex is the most convenient form of Hb for estimating the relative contents of the heme orientational isomers from its proton NMR spectrum. In addition, conversion from disordered to native heme orientation isomer is relatively slow in this form (La Mar et al., 1984). This was done for all the ferric reconstituted Hbs, except metcyanodeuteroHb, by adding NaN_3 within 5 min after the recombination. The heme orientation for metcyanodeuteroHb was determined on the basis of the β-pyrrole proton resonances. Reconstituted HbCO was converted to the metazido complex by adding ferricyanide and sodium azide.

NMR Measurements. Proton NMR spectra at 300 MHz were recorded on a Nicolet NT-300 spectrometer equipped with a 1280 computer system. Typically, a 13-μs (180°) radio-frequency pulse and a $\pm$12-kHz spectral width were used to detect the heme methyl signal for reconstituted hemoglobins (5–30 ppm from H_2O). A conventional WEFT pulse sequence (180°–τ–90° acuire) 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. Usually, an interpulse time of about 0.1 s was used (Inubushi et al., 1983). Proton shifts were referenced with respect to the water proton signal, which was 4.8 ppm downfield from the proton resonance of 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 23 °C.

Results and Discussion

Heme Peripheral Effect on the Heme Orientation. Figure 2 shows the hyperfine-shifted portion ^{1}H NMR spectra of sperm whale deuterohemin–metMbN_3^- that was obtained by the reconstitution reaction of apoHb with twice a stoichiometric amount of deuterohemin–azido complex. The spectrum recorded immediately after reconstitution (Figure 2A) consists of two sets of heme methyl resonances. By referring to the previously reported spectra for the reconstituted metazido protoMb, metcyanoprotoMb (Mayer et al., 1974; La Mar et al., 1981, 1983), and metcyanodeuteroMb (La Mar et al., 1978) and the time dependences of these spectra, the heme methyl peaks M_1, M_2, and M_3 can be assigned to the native heme orientation isomer of deuteroMb. The peaks M'_1, M'_2, M'_3, and M'_4, which decreased in their intensities with time (Figure 2B), can readily be assigned to the heme disordered form. Traces C and D in Figure 2 illustrate the spectra for metazidodeuteroHb obtained 15 min and 2 days after reconstitution, respectively. Our previous report (Ishimori & Morishima, 1986) has shown that the methyl resonances la-

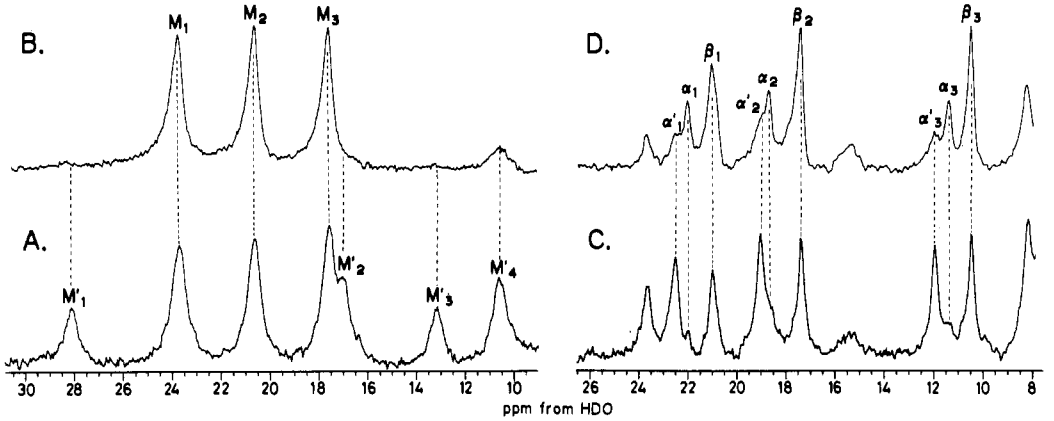

FIGURE 2: Downfield hyperfine-shifted portions of the 300-MHz ^{1}H NMR spectra of reconstituted deuteroMb and -Hb as the metazido complex in 50 mM Bis-Tris, pH 7.0 at 23 °C; reconstituted deuteroMb (A) 15 min and (B) 2 days after reconstitution and reconstituted deuteroHb (C) 15 min and (D) 2 days after reconstitution.

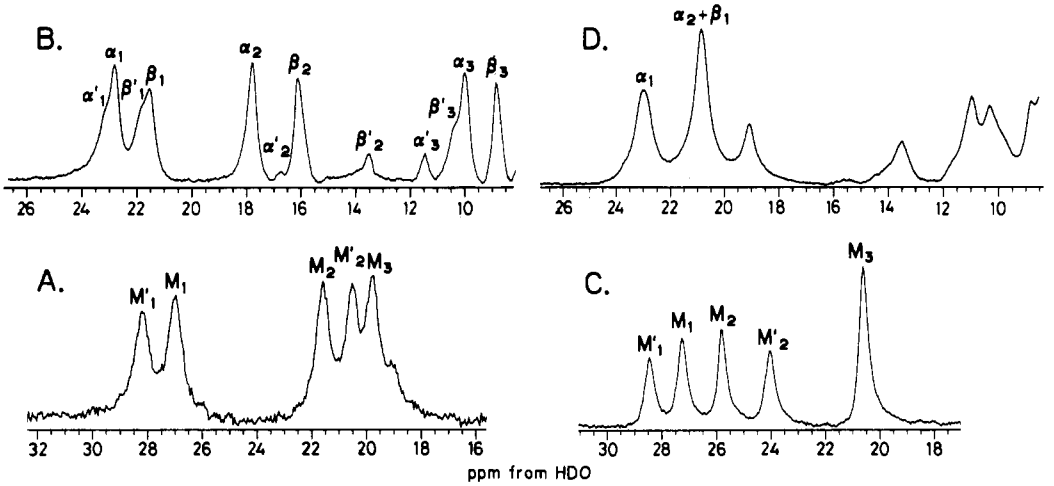

FIGURE 3: Downfield hyperfine-shifted portions of the 300-MHz ^{1}H NMR spectra of reconstituted protoMb and -Hb and mesoMb and -Hb as the metazido complex taken 30 min after reconstitution in 50 mM Bis-Tris, pH 7.0 at 23 °C: (A) Reconstituted protoMb; (B) reconstituted protoHb; (C) reconstituted mesoMb; (D) reconstituted mesoHb.

beled α'_n and β_n (n = 1–3) are attributed to the α and β subunit, respectively. In addition to the six peaks in the NMR spectrum taken immediately after the reconstitution (Figure 2, trace C), we find three additional peaks labeled α_1, α_2, and α_3, which have larger intensities than the three methyl peaks α'_1, α'_2, and α'_3 in trace D. Intensities of the peaks α_n (n = 1–3) increase in time at the expense of the peaks α'_n (n = 1–3), whereas the peaks β_1, β_2, and β_3 exhibit no significant time-dependent changes. It is therefore likely that the species affording the peaks α'_n (n = 1–3) is metastable and converts slowly to the form that generates the peaks α_n (n = 1–3). This indicates that the protein conformation formed initially is not stable and that two forms of the protein conformation are present 2 days after the reconstitution. The presence of these two sets of peaks appears similar to that found for freshly reconstituted deuteroMb as shown in Figure 2A. On the basis of the time-dependent spectral changes for reconstituted deuteroMb and -Hb, it is concluded that the metastable component giving the peaks α'_n (n = 1–3) bears the disordered heme orientation, whereas the resonances α_n and β_n (n = 1–3) arise from the native heme orientation. Therefore, upon incorporation of deuterohemin–N_3^-, the apoHb α subunit prefers the disordered heme orientation, while the β subunit prefers the native orientation.

Jue and La Mar (1984) reported that the heme disorder exists in both α and β subunits of deoxygenated and metcyanodeuteroHbs. We previously found (Ishimori & Morishima, 1986) that the "reversed" heme reorientation reaction occurs from the native to disordered form when the ferric azido deuteroHb is reduced to the ferrous deoxygenated form. Therefore, the disordered form that Jue and La Mar (1984) observed for the deuteroHb β subunit appears to originate from the native form by the reversed reorientation conversion.

Figure 3 shows the ^{1}H NMR spectra of the azido complexes of reconstituted protoMb(A), protoHb(B), mesoMb(C), and mesoHb(D). In trace A, the three methyl signals M_1, M_2, and M_3 are assigned to the native heme orientation, and the two methyl signals M'_1 and M'_2 are assigned to the disordered form, as judged from the time course of the NMR spectra. For the reconstituted protoHb, the preferential azide binding to the β subunit of metazidoHb A (Gibson et al., 1969) and the time-course spectra (La Mar et al., 1985) have led to the assignments of the α_1, α_2, α_3, β_1, β_2, and β_3 methyl resonances to the native heme orientation for the α and β subunits (Neya & Morishima, 1981) and the α'_1, α'_2, α'_3, β'_1, β'_2, and β'_3 resonances to the disordered one for the α and β subunits, respectively (Yamamoto & La Mar, 1986). The use of the methyl-deuteriated heme for the reconstituted Hb and Mb allowed La Mar et al. (1983, 1985) to assign individual methyl resonances for protoMb as M_1 (5-CH_3), M_2 (1-CH_3), and M_3 (8-CH_3) and those for protoHb as α_1, β_1 (5-CH_3), α_2, β_2 (1-CH_3), and α_3, β_3, (8-CH_3). The spectrum for the reconstituted mesoMb (trace C) also exhibits two sets of heme methyl resonances arising from the two heme orientations, the signals M_1, M_2, and M_3 from the native heme orientation and the peaks M'_1 and M'_2 from the disordered one. On the other

Table I: Proportions of Native Orientation Relative to Disordered Orientation in the Reconstitution Reaction between Hemin–Azide Complex and ApoMb and ApoHb in 50 mM Bis-Tris, pH 7.0 at 23 °C

	myoglobin	hemoglobin α	hemoglobin β
deuterohemin	7:3	0:10	10:0
protohemin	5:5	7:3	6:4
mesohemin	6:4	10:0	10:0

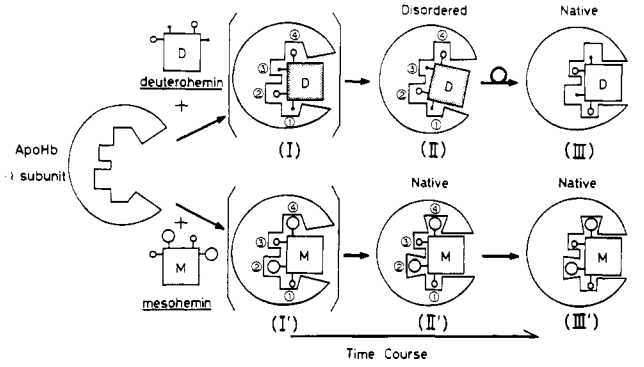

FIGURE 4: Schematic representations of the mechanism of the specific heme orientation (see text).

hand, as shown in trace D, mesoHb consists of only one component. Signal α_1 has been assigned to the α subunit and the peak $\alpha_2 + \beta_1$ to the combined signals arising from the α and β subunits (Neya & Funasaki, 1986). These results indicate that both Mb and Hb reconstituted with protohemin and the reconstituted mesoMb contain two heme orientation isomers, whereas the reconstituted mesoHb favors only one heme orientation. The relative proportions of the two orientation isomers in Hb and Mb reconstituted with metazidohemin complex are assembled in Table I.

The present results of the proton NMR spectra of reconstituted Hbs have revealed that the heme orientation depends on the heme peripheral 2,4-substituents (Figures 2 and 3 and Table I). Since the apoHb α subunit combines with the deuterohemin–N_3^- preferentially in the disordered heme orientation, the apoHb α subunit can sense the difference in the steric bulkiness between heme methyl groups at the 1,3-position and protons at the 2,4-position when heme is embedded in the heme crevice. The preferential native heme orientation for mesoHb suggests that apoHb can also distinguish stereochemically between the methyl and ethyl groups at the heme periphery. However, as apoHb initially accommodates protohemin–N_3^- in two orientations equally, apoHb cannot stereochemically distinguish between the vinyl and methyl groups. It is therefore likely that apoHb recognizes the heme peripheral substituents at the early stage of the incorporation reaction, thereby determining the specific heme orientation taken up.

These findings allow us to propose a mechanism for the stereospecific heme incorporation into the apoHb α subunit as schematically shown in Figure 4. When the heme is incorporated into the heme cavity, it is proposed that the apoHb α subunit and hemin form an intermediate complex (I, I') in which apoHb recognizes the relative steric bulkiness of the substituents at the heme positions 1–4. The sites 1–4 in Figure 4 are defined as follows. For native Hb, the vinyl groups at the 2,4-position of protohemin are located at sites 2 and 4, while the methyl groups at the 1,3-position are at sites 1 and 3. This heme orientation is referred to as the native heme orientation. In the intermediate complex, the apoHb α subunit orients the hemin in a position where the bulkier groups occupy sites 2 and 4 as shown in the figure. Since deuterohemin (1,3-CH_3, 2,4-H) bears bulky groups at the 1,3-position, the heme orientation has to be disordered by a 180° rotation about the α,γ-meso axis (I). On the other hand, mesohemin (1,3-CH_3, 2,4-CH_3CH_2), with much bulkier groups at the 2,4-position, is embedded in the native orientation (I').

After the recombination reaction is completed, slow change of the heme orientation is observed for deuteroHb and protoHb, implying that the heme orientation selected during complex I formation becomes metastable in complex II. It is of interest that this second step occurs for deuteroHb and in the disordered protoHb subunit, in which the heme sites 1 and 3 are occupied by the proton and the vinyl group, respectively. Sites 1 and 3 for mesoHb and the native protoHb α subunit that did not exhibit a heme orientational conversion are occupied by the methyl group. It is therefore tempting to suggest that after the intermediate complex is converted to the stable complex (II, II'), the sites which recognize the methyl groups may be formed on the protein at sites 1 and 3. For the deuteroHb α subunit in which apoHb initially combines with the disordered deuterohemin, with sites 1 and 3 occupied by protons, the disordered form is slowly converted to the more stable complex (III) with the native heme orientation, in which sites 1 and 3 are occupied by methyl groups. On the other hand, the α subunit of apoHb initially combines with the mesohemin in the native heme orientation, with sites 1 and 3 already occupied by methyl groups, and therefore does not experience further conversion of heme orientation. As for protohemin–N_3^-, which reacts with apoHb to afford two heme orientation isomers, the disordered heme orientation is slowly converted to the native heme orientation at the second stage. This implies that the heme orientation may be determined at two independent stages (stages I and II in Figure 4).

As for the apoHb β subunit, the mechanism could be somewhat different from that for the α subunit. Deuterohemin–N_3^- is initially inserted to apoHb β subunit in one orientation so that the protein structure senses the relative steric difference between the proton and the methyl group at the heme periphery, as found for apoHb α subunit. However, the deuteroHb β subunit prefers the native heme orientation in contrast to the disordered heme orientation for the α subunit. Mesohemin–N_3^- favors the native heme orientation in mesoHb β subunit, as also found in the α subunit. It is suggested that at the first step of the determination process for the heme orientation in the apo β subunit its protein conformation does not orient the heme in favor of less bulky groups located at sites 1 and 3, as found for the α subunit. Rather, it favors the heme oriented in such a way that methyl groups occupy sites 1 and 3, as shown in Figure 4. That is, protein recognizes the methyl groups at sites 1 and 3 immediately after apoHb combines with heme. Nonstereospecific heme orientation in the β subunit for the reconstitution reaction with protohemin–N_3^- implies that at the first stage the β subunit cannot recognize the difference between the vinyl and the methyl groups. Since the disordered form is converted to the native form in the reconstituted protoHb, the protein may recognize the difference between the vinyl and the methyl groups in the β subunit at the second stage. Therefore, the heme orientation is determined at two stages for both apo α and β subunits.

Heme Ligand Effect on the Orientation. In order to look at the effect of the axial heme ligand on the heme orientations, the recombination reaction of apoHb, with the hemin complexes coordinated with a variety of axial ligands, was followed by NMR. Figure 5 compares the NMR spectra of deuteroHb–N_3^-, which is formed either by reconstitution with deuterohemin–N_3^- (A) or by reconstitution with deuterohemin–

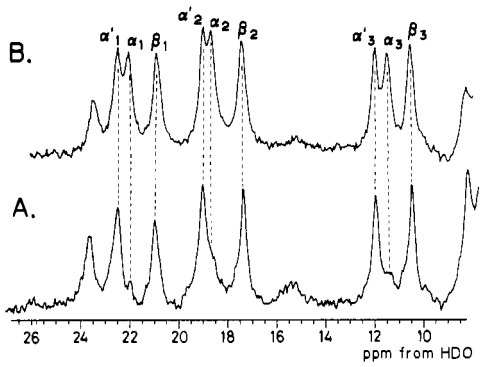

FIGURE 5: Downfield hyperfine-shifted portions of the 300-MHz ^1H NMR spectra of reconstituted deuteroHb as the metazido complex taken 30 min after reconstitution in 50 mM Bis-Tris, pH 7.0 at 23 °C: (A) Reconstituted with deuterohemin–N_3^-; (B) reconstituted with deuterohemin–pyridine.

Table II: Proportions of Native Orientation Relative to Disordered Orientation in the Reconstitution Reaction between α and β Subunits of ApoHb and Some Hemin Derivatives Coordinated with a Variety of Axial Ligands in 50 mM Bis-Tris, pH 7.0 at 23 °C

axial ligand	H_2O	CO	N_3^-	CN^-	pyridine
		(A) α Subunit			
deuterohemin	0:10	0:10	0:10	4:6	5:5
protohemin	7:3	5:5	7:3	*a*	6:4
mesohemin	10:0	10:0	10:0	*a*	4:6
		(B) β Subunit			
deuterohemin	10:0	10:0	10:0	5:5	10:0
protohemin	6:4	5:5	6:4	*a*	5:5
mesohemin	10:0	10:0	10:0	*a*	5:5

[a] Not determined.

pyridine followed by subsequent addition of NaN_3 (B). The deuterohemin–pyridine complex incorporates into apoHb to afford two orientation isomers for the α subunit as seen by the signals α_1, α_2, and α_3 arising from the native heme orientation and the α'_1, α'_2, and α'_3 peaks from the disordered one but only one heme orientation conformer for the β subunit (the signals β_1, β_2, and β_3 due to the native orientation). This heme orientation behavior for the α subunit differs from the case in which azidohemin is directly incorporated into apoHb. Mesohemin–pyridine incorporates into the α and β subunits in two heme orientations (Table II), while the azido complex is inserted in a different way. It follows that the heme orientation depends not only on the heme substituents but also on the heme axial ligand.

Figure 5 and Table II show that the stereospecific recognition of the heme orientation as illustrated in Figure 4 is substantially influenced by the axial heme ligand. When bis(pyridine) or bis(cyano) complexes of hemin are inserted into apoHb, nearly equal percentages of the two heme orientations are seen. In the case where the reconstitution reaction is performed between deuterohemin–pyridine and the β subunit, the percentages are different. This may suggest that at the first stage of recombination there is no specific recognition by apoprotein of the heme orientation when the axial ligand is rather bulky and/or when hemin is bis coordinated. Nonbonded interactions between globin and the iron-bonded ligand(s) may be more important than the globin–heme peripheral group interactions, leading to nonstereospecific recognition of the heme orientation irrespective of the 2,4-substituent.

Effect of Apoprotein Structure on the Heme Orientation. In an effort to determine the effect of the apoprotein structure on the heme orientation, we examined the reconstitution by utilizing semiHbs that contained heme in only one subunit.

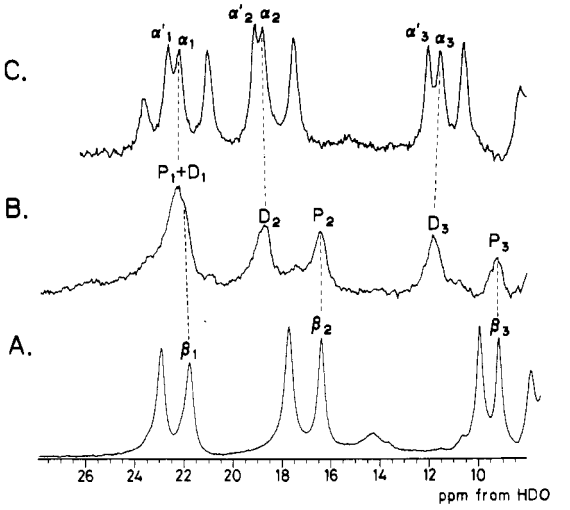

FIGURE 6: Downfield hyperfine-shifted portions of the 300-MHz ^1H NMR spectra of native Hb, semiHb, and reconstituted deuteroHb as the metazido complex in 50 mM Bis-Tris, pH 7.0 at 23 °C: (A) Native Hb; (B) semiHb β taken 30 min after addition of deuterohemin–N_3^-; (C) deuteroHb reconstituted with deuterohemin–pyridine complex.

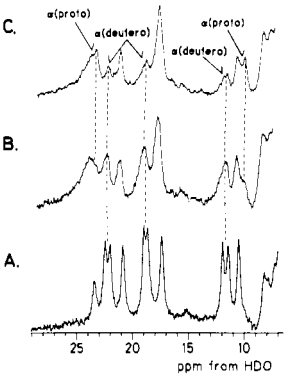

FIGURE 7: Heme-exchange reaction as followed by 300-MHz ^1H NMR spectra of the metazido complex of deuteroHb in 50 mM Bis-Tris, pH 7.0 at 23 °C: (A) In the absence of protohemin–N_3^-; (B) 1 h after addition of protohemin–N_3^-; (C) 12 h after addition of protohemin–N_3^-.

In Figure 6B is shown the NMR spectrum taken immediately after addition of the deuterohemin–N_3^- to semiHb β that contained protoheme only in the β subunit. Figure 6A illustrates the NMR spectrum of native Hb–N_3^-. As a reference, the spectrum of deuteroHb–N_3^-, which was reconstituted with deuterohemin–pyridine followed by addition of N_3^-, is shown in Figure 6C. Upon addition of deuterohemin–N_3^- to semiHb β, the new peaks grow at 18.5 (D_2) and 11.5 (D_3) ppm, due to deuteroHb α subunit in the native heme orientation. The resonance P_1+D_1, which has an intensity two times as large as the resonances D_2 or P_2, presumably originates from the methyl signals (β_1 and α_1) arising from proto- and deuteroheme, respectively. In other words, the resonance P_1+D_1 does not resolve into α_1 and β_1 in this case.

To gain further insight into the effect of heme incorporation on heme orientation in the complementary subunit, a heme-exchange reaction was performed. In Figure 7, the time-dependent spectral changes for deuteroHb–N_3^-, after protohemin–N_3^- is added, are illustrated. Parts A, B, and C of Figure 7, respectively, show the spectra before, 1 h after, and 12 h after the addition of protohemin–N_3^-. The resonances at 22.5, 22.0, 18.9, 18.7, 11.9, and 11.3 ppm, assignable to the deuteroHb α subunit, decrease in their relative intensities with time, and the new peaks are observed at 23.0 and 9.7 ppm.

Table III: Proportions of Native Orientation Relative to Disordered Orientation in the Reconstitution Reaction between Hemin–Azide Complex and SemiHbs and in the Heme-Exchange Reaction in 50 mM Bis-Tris, pH 7.0 at 23 °C

	deuterohemin		protohemin		mesohemin	
	α	β	α	β	α	β
apoHb	0:10	10:0	7:3	6:4	10:0	10:0
semiHb α		10:0		10:0		10:0
semiHb β	10:0		6:4		10:0	
heme exchange[a]			10:0	NR[b]		

[a] Exchange from deuteroheme to protoheme in deuteroHb–N_3^-. [b] No reaction.

The resonance positions of these new peaks coincide with those for the native heme orientation isomer in the protoHb α subunit. However, the resonances due to the deuteroHb β subunit exhibit no significant changes. The results of the reconstitution reactions of semiHbs with some hemin derivatives are compiled in Table III.

As shown in Table III, the order of specificity of the heme orientation is as follows: α subunit for the heme exchange reaction > β subunit in semiHb α > α subunit in semiHb β > β subunit in apoHb > α subunit in apoHb > apoMb.

It has been revealed that apoMb is monomeric, while semiHb β and apoHb are dimeric and semiHb α is tetrameric (Cassoly & Banerjee, 1971; Winterhalter et al., 1968). The specificity of the heme orientation mentioned above appears to parallel the extent of the protein aggregation (semi Hb α > semi Hb β, apo Hb > apo Mb). This implies that a subunit–subunit interaction in Hb renders the heme orientation stereospecific. The NMR resonances of the hydrogen-bonded protons (α126H8 Asp–β35C1 Tyr and α103G10 His–β108G10 Asn) (Russu et al., 1987), which are located at the $\alpha_1\beta_1$ intersubunit interface, are observed in apoHb, semiHb α, and semiHb β (Ishimori and Morishima, unpublished data). This suggests that these Hbs containing heme-free subunits maintain their quaternary structures by intersubunit hydrogen bonds. Quaternary structure could affect the tertiary structure of an apo subunit so that recognition of the heme orienation is highly stereospecific.

It is noted that heme is more stereospecifically inserted into the apo subunit of semiHb derivatives having the heme embedded in the complementary subunit than into apoHb with all subunits free of heme. As shown in Table III, apoHb exhibits the lowest specificity. SemiHbs exhibit a higher specificity. The highest specificity of the heme orientation is exhibited in the heme-exchange reaction where the heme is inserted into one subunit of the Hb molecule with the other three subunits occupied by heme. As heme incorporation stabilizes the tertiary structure of the apoprotein conformation, it could give rise to the same effects on the steeospecific heme orientation as those exerted by aggregation of the subunit. From the above discussion, it is suggested that the heme orientation could be determined by the protein conformational flexibility, which is modulated by the formation of the intersubunit hydrogen bonds and/or by the incorporation of the heme.

The effect of the apoprotein structure on the heme orientation is also noticed for α and β subunits of apoHb and semiHb. Table III shows that the β subunit exhibits higher specificity in the heme orientation than the α subunit. It is likely that this difference between the α and β subunits arises from the relative differences in their heme environmental structures. We previously reported (Ishimori & Morishima, 1986) that the modification of the heme side chains in the β subunit exerts a more subtle structural alteration than does heme side-chain modification in the α subunit. These nonequivalent structural changes induced by the heme substitution between the α and β subunits have also been encountered for metal-substituted Hb and its hybrid Hb derivatives (Inubushi et al., 1983; Shibayama et al., 1987; Ishimori & Morishima, 1988). This nonequivalence may arise from the substantial difference in van der Waals contacts in heme environments between the α and β subunits; the van der Waals contacts in the β subunit are more substantial than in the α subunit. The stronger interactions between heme and globin in the β subunit bring about more stereospecific recognition of the heme orientation in the β subunit than in the α subunit.

In summary, in the present NMR study we have found factors that control the heme orientation at reconstitution, leading to a proposal of a mechanism for the determining process of heme orientation (Figure 4). ApoHb senses the steric difference of both the porphyrin 2,4-substituents and the axial iron ligand in the heme–apoprotein recombination reaction; the process determining heme orientation consists of the two stages. Furthermore, subunit structural changes propogated from the complementary subunit through subunit–subunit interactions are quite sensitively sensed by the stereospecific heme orientation, suggesting that the intersubunit interactions play an important role in the stereospecific recognition of the heme incorporation into apoproteins.

Registry No. Deuteroheme, 18922-88-8; protoheme, 14875-96-8; mesoheme, 18040-04-5.

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Amino Acid Sequences of Substrate-Binding Sites in Chicken Liver Fatty Acid Synthase†

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ABSTRACT: The amino acid sequences of three essential regions of chicken liver fatty acid synthase have been determined: that around 4′-phosphopantetheine ("carrier" site), the substrate "loading" site containing serine, and a "waiting" site for the growing fatty acid containing cysteine. The amino acid sequence of the 4′-phosphopantetheine region was determined for the acetyl-, malonyl-, hydroxybutyryl-, and butyryl-enzyme with peptides obtained by hydrolysis of the enzyme with trypsin and *Staphylococcus aureus* (V8) protease. The sequence region around the essential serine was obtained for the acetyl- and malonyl-enzyme. The N-terminus of the tryptic peptide was blocked. However, the same sequence is obtained for the acetyl- and malonyl-peptide after *S. aureus* protease digestion, suggesting that the enzyme contains a single acyl transferase rather than two separate transacylases. The sequence around the cysteine was obtained by use of a radioactive iodoacetamide label. An unusual sequence of three serines adjacent to the cysteine was found. The strong similarities between peptides from different species for all three of the regions suggest that the multifunctional polypeptides from yeast and animals have evolved from the monofunctional enzymes of lower species.

Chicken liver fatty acid synthase (M_r ~500 000) is one of the most complex multienzyme complexes in the animal kingdom: It consists of two identical polypeptide chains [cf. Wakil et al. (1983)] and contains six different enzyme activities. The enzyme catalyzes the synthesis of palmitic acid according to the overall reaction

$$\text{acetyl-CoA} + 7\text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{palmitic acid} + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O} + 7\text{CO}_2$$

The mechanism involves initiation of the fatty acid chain by transfer of an acetyl group to the enzyme from acetyl-CoA[1] (acyl transferase). The chain is lengthened by the transfer of a malonyl group to the enzyme from malonyl-CoA (acyl transferase). The condensation of acetyl and malonyl gives rise to the acetoacetyl-enzyme (β-ketoacyl synthase); this enzyme-bound intermediate is reduced to 3-hydroxybutyryl-enzyme by NADPH (β-ketoacyl reductase); dehydration gives the crotonyl-enzyme (dehydratase), which is reduced to butyryl-enzyme by NADPH (enoyl reductase). This cycle is repeated a total of 7 times, with transfer of a malonyl moiety to the enzyme initiating each cycle. After the thioester of palmitic acid is formed, the free fatty acid is released into solution (thioesterase). The overall and elementary steps in the reaction mechanism, the detailed stereochemistry, the distribution of reaction intermediates on the enzyme, and the distances between several specific sites have been explored [cf. Hammes (1985), Cox and Hammes (1983), Cognet and Hammes (1983, 1985), Anderson and Hammes (1984, 1985), Yuan and Hammes (1985, 1986), and Chang and Hammes (1986)].

The structure of the enzyme has been probed by limited proteolysis and chemical modification [cf. Wakil et al. (1983)]. The two polypeptides are arranged head-to-tail with two independent catalytic centers, each derived from two different polypeptide chains. Limited proteolysis yields three polypeptides on sodium dodecyl sulfate–polyacrylamide gels (Tsukamoto et al., 1983). Peptide I contains the acyl transferase, the serine utilized as a substrate "loading" site, and the cysteine that serves as a "waiting" site by forming a thioester with the growing saturated fatty acid chains while a malonyl moiety is loaded onto the enzyme. Peptide II contains the dehydratase, the reductases, and the 4′-phosphopantetheine to which the substrate is bound while the synthase, dehydratase, reductases, and thioesterase act on it. Peptide III is the thioesterase. The β-ketoacyl synthase and β-ketoacyl reductase enzymes require both polypeptide chains for activity,

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[1] Abbreviations: CoA, coenzyme A; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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