Molecular Recognition of Horse Heart Apomyoglobin to Monopropionate Hemin: Thermodynamic Determination of Two Orientational Isomers by ^{1}H NMR Spectra

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(Received July 18, 1995)

Four synthetic hemins, 1,3,5,8-tetramethyl-2,4-diethyl-6,7-dipropionate hemin (**1**), 1,2,3,4-tetraethyl-5,6,8-trimethyl-7-propionate hemin (**2**), 1,3,5,6,8-pentamethyl-2,4-diethyl-7-propionate hemin (**3**) and 1,3,5,7,8-pentamethyl-2,4-diethyl-6-propionate hemin (**4**) were prepared and incorporated into apomyoglobin to clarify hydrophilic interaction of the hemin propionate with particular polar amino acid residues. Favorable orientation of hemin in protein is interpreted by thermodynamic study using ^{1}H NMR measurement.

Interaction between apoprotein and prosthetic hemin is related to one of the current chemistry of molecular recognition in biological systems.[1] Chemical modification of the hemin provides us an alternative approach to understand specific molecular recognition in the hemoprotein in place of site-directed mutagenesis of apoprotein. In particular, interaction of two propionates at the close proximity to the spherical protein surface seems to play an important role in stabilization of the deoxy and oxy forms via salt bridges and/or hydrogen bonds.[2,3] According to structural analysis of horse heart myoglobin (Mb) by X-ray crystallography, 6- and 7-propionates of hemin interact with Lys45 and Ser92/His97, respectively, and these specific sites and several water molecules associate to form the unique salt-bridge (hydrogen bond) network to stabilize hemin conformation.[4] In contrast, a set of van der Waals contacts between hydrophobic protein residues and 1,2,3 and 4-positions of peripheral methyl and vinyl groups affects hemin orientation considerably. Recognition of axially asymmetric hemin in the chiral cavity of the protein brings about the two orientational isomers via a variety of interactions with amino acid residues. We have already reported X-ray crystallographic studies and physiological properties of the reconstituted Mbs to discuss the substitution effect of 1-4 positions on the heme orientation.[5] In this paper, we focus on the thermodynamic aspects of the synthetic hemin-protein contacts by ^{1}H NMR spectroscopy.

To simplify the binding fashion in heme pocket, we prepared four synthetic hemins: 1,3,5,8-tetramethyl-2,4-diethyl-6,7-dipropionate hemin (**1**), 1,2,3,4-tetraethyl-5,6,8-trimethyl-7-propionate hemin (**2**), 1,3,5,6,8-pentamethyl-2,4-diethyl-7-propionate hemin (**3**) and 1,3,5,7,8-pentamethyl-2,4-diethyl-6-propionate hemin (**4**).[6] These synthetic hemins were incorporated into apoMb from horse heart by usual method.[7] Throughout this paper, Mbs reconstituted with hemin **1**-**4** are abbreviated as rMb(**1**)-rMb(**4**), respectively. Each reconstituted Mb is characterized by electronic absorption[8] and ^{1}H NMR spectra. Table I shows ^{1}H NMR chemical shifts of several peaks of the peripheral methyl protons and γ- and δ-protons of Ile99 in cyano complex at ambient temperature after reaching equilibrium. These peaks are assignable according to the earlier reports by La Mar and his co-workers.[9] These spectra reveal the presence of two sets of resonances due to not the existence of two enzyme structures but the two interconverting heme orientations by a 180° rotation about the α-γ-meso axis in heme pocket. The ratio of major/minor components is determined by the peak area calculated from curve fitting analysis of Lorentzian lineshapes in the region of Ile99 signals. Furthermore, we can assign the favorable orientation determined by number of characteristic methyl signals, which appear at down-field region (> 20 ppm), since substituted methyl signals at 1,2,5 or 6 positions of hemin ring shift to down field compared with those of the mirror orientation. Each favorable orientation for **1**-**4** sets is shown at the left side of Figure 1, where the number of the peripheral positions are designated clockwisely from overview of the proximal histidine (His97).

Major/minor interconvertible ratio of heme orientation in heme pocket at equilibrium is governed by thermodynamic parameters involving the various specific weak non-covalent interactions between hemin and amino acid residues. It is assumed that, firstly, conformational change of the protein is small upon incorporation of the synthetic hemin and secondly free energy change of the heme-protein interaction can be separated into part of hydrophobic interaction ($\Delta G°_N$ or $\Delta G°_R$) and two parts of hydrophilic interactions ($\Delta G°_6$ and $\Delta G°_7$) at Lys45 and Ser92/His97 sites as shown in Figure 1. Equilibrium ratio of major and minor orientations determined from ^{1}H NMR gives rise to the free energy difference ($\Delta\Delta G°$) between two Mbs incorporated with normally oriented hemin and reversed one. The peripheral 1- and 3-methyl groups of mesohemin **1** in rMb(**1**) is superimposable with those of protohemin in native Mb, and normal orientation is more stable than the reversed orientation in rMb(**1**), thus $\Delta\Delta G°$ in rMb(**1**) is expressed as

$$\Delta\Delta G°_1 = \Delta G°_N - \Delta G°_R = -1.2 \text{ kcal/mol (observed)} \quad (1)$$

where $\Delta G°_N$ and $\Delta G°_R$ are free energy changes upon interaction of hydrophobic amino acid with peripheral alkyl groups in

Table 1. 500 MHz ^{1}H NMR chemical shifts of reconstituted horse heart metcyanomyoglobins in H_2O-D_2O, pH 7.0 at equilibrium[a]

myoglobin	methyl[b] major, minor		Ile99[c] major, minor		ratio[d] (major / minor)
rMb(**1**)•CN	25.0	25.1	-3.0	-2.6	7.1
	21.6	19.7	-3.5	-3.1	
			-8.3	-6.7	
rMb(**2**)•CN	24.9	21.6	-2.3	-2.0	2.6
	22.5		-2.6	-2.6	
			-5.2	-4.4	
rMb(**3**)•CN	25.6	22.6	-2.9		29
	22.0	20.9	-3.2		
	21.4		-7.5	-5.3[e]	
rMb(**4**)•CN	22.5	26.2	-2.6	-2.5	3.8
	20.5	22.8	-3.2	-2.9	
		20.0	-6.8	-6.2	

[a]Shifts in ppm from DSS in D_2O solution at 25 °C. [b]Peripheral methyl protons of hemin (1-8 Me). [c]γ- and δ-protons of Ile99. [d]Ratio of peak areas between major and minor components calculated from Ile99 protons. [e]Minor component was not completely detected by ^{1}H NMR spectrometer.

normal and reverse components, respectively, in Figure 1. Moreover, interaction of a propionate of **2** with Ser92/His97 constitutes more stable form in rMb(**2**).

$$\Delta\Delta G^\circ_2 = \Delta G^\circ_7 - \Delta G^\circ_6 = -0.57 \text{ kcal/mol (observed)} \quad (2)$$

where ΔG°_7 and ΔG°_6 are free energy changes upon interaction of 7-propionate-Ser92/His97 and 6-propionate-Lys45 contacts, respectively, in Figure 1. These results support that the peripheral alkyl groups predominate favorable orientation of hemin in protein compared with polar side chain at 6 and 7-positions.

For rMb(**3**) and rMb(**4**), hemin **3** and **4** have asymmetric hydrophobic groups and asymmetric hydrophilic propionate on rotation around α-γ axis. The stability differences in two orientational isomers in rMb(**3**) and rMb(**4**), $\Delta\Delta G^\circ_3$ and $\Delta\Delta G^\circ_4$, are experimentally observed by -2.0 and -0.79 kcal/mol, respectively. It is possible to estimate the differences of free energy changes for rMb(**3**) and rMb(**4**) from combination of $\Delta\Delta G^\circ_1$ and $\Delta\Delta G^\circ_2$, if both hydrophobic and hydrophilic interactions between hemin and protein are independent as are shown in the equations (3) and (4).

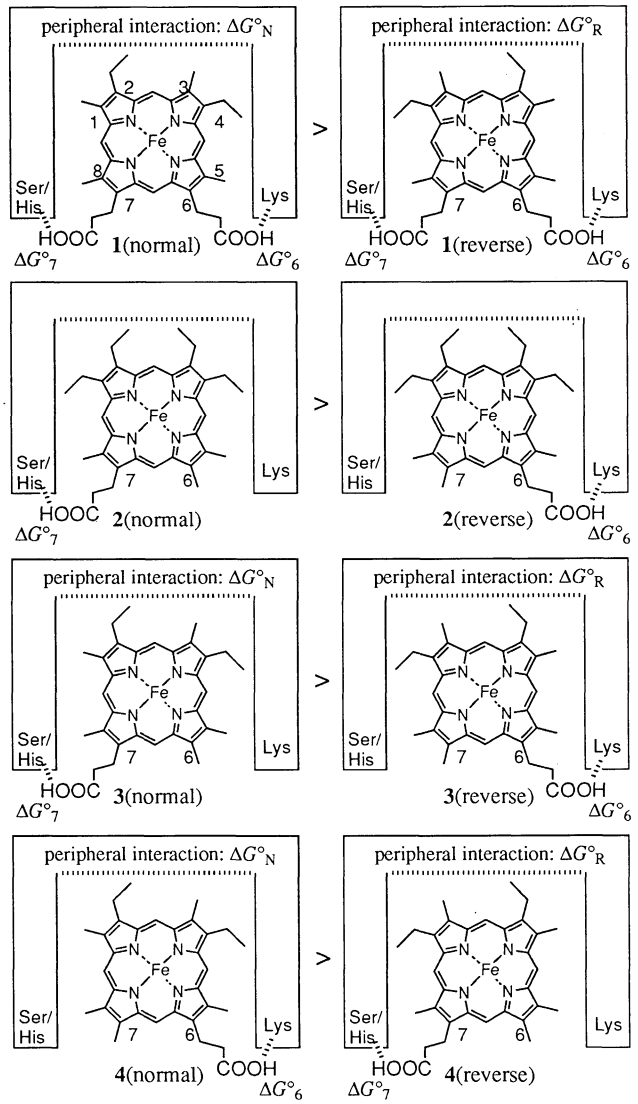

Figure 1. Schematic representation of two orientational isomers of hemin **1-4** in heme pocket. According to x-ray structural analysis, Lys45 and Ser92/His97 contact with 6- and 7-propionates of hemin, respectively. Free energy changes upon hydrophilic interaction with these amino acid residues are defined as ΔG°_6 and ΔG°_7, respectively. In contrast, free energy changes upon interaction between hydrophobic residues and heme peripheral alkyl groups at **1-4** positions in normal and reverse forms are defined as ΔG°_N and ΔG°_R, respectively.

$$\Delta\Delta G^\circ_3 = (\Delta G^\circ_N + \Delta G^\circ_7) - (\Delta G^\circ_R + \Delta G^\circ_6) = -1.8 \text{ kcal/mol (calculated)} \quad (3)$$

$$\Delta\Delta G^\circ_4 = (\Delta G^\circ_N + \Delta G^\circ_6) - (\Delta G^\circ_R + \Delta G^\circ_7) = -0.63 \text{ kcal/mol (calculated)} \quad (4)$$

The calculated $\Delta\Delta G^\circ_3$ and $\Delta\Delta G^\circ_4$ are comparable to those obtained from ^{1}H NMR studies. This result demonstrates the additivity of the stability differences consisting of each independent free energy changes of interaction between peripheral alkyl groups and amino acid residues.

Finally, we estimate the favorable orientation of synthetic hemin via specific interactions in protein by thermodynamic parameters of simple and known heme-protein contacts. The reconstitution of apoprotein with synthetic hemins provides us good tool to understand dynamic molecular recognition of prosthetic group in protein as an alternative to an approach of site-directed mutagenesis of apoprotein. Further work on physiological properties of the presented reconstituted Mbs is in progress.

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