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Heme methyl hyperfine shift pattern as a probe for determining the orientation of the functionally relevant proximal histidyl imidazole with respect to the heme in hemoproteins

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Heme methyl ^1H and ^{13}C resonances of met-cyano form of myoglobin from the shark, *Geleorhinus japonicus* (GJMbCN), have been assigned via ^1H-^{13}C heteronuclear shift correlated spectroscopy (COSY) connectivities and their hyperfine shifts were compared with those of the corresponding resonances of some hemoproteins. Variation of the heme methyl ^1H hyperfine shift pattern correlates well with the angle (Φ) between the projection of the proximal histidyl imidazole plane onto the heme plane and the N_{II}-Fe-N_{IV} vector. The alteration of the interaction of the heme peripheral side-chains and/or the iron-bound ligand with the surrounding amino acid residues cannot account for large differences in the shifts of the corresponding heme methyl resonances between GJMbCN and sperm whale MbCN. Since the heme methyl ^1H shifts for GJMbCN fall in between those of the corresponding resonances for sperm whale Mb and *Aplysia limacina* Mb in which the Φ values have been reported to be 19° and 29°, respectively, the Φ value in GJMb is estimated to be slightly larger than 19°.

NMR; Myoglobin; Heme methyl resonance; Heme electronic structure; Proximal histidyl imidazole orientation

1. INTRODUCTION

The nature of the bonding interaction between the heme iron and the proximal histidyl imidazole is known to play a central role in controlling the reactivity of the active center of hemoproteins [1]. A minor alteration in this bonding interaction leads to drastic changes in the electronic structure of the heme (structure in Fig. 1) and hence the reactivity of the heme iron. The g-tensors have been used to characterize quantitatively the influence of the axial ligand orientation on the heme electronic structure, because the interaction of the imidazole P_π orbital with the partially filled d_π orbital of the center iron is mainly modulated by the crystal field parameters [2–6]. NMR spectroscopy has also provided useful information about the active site of the hemoproteins [7]. Especially the hyperfine shifted heme methyl ^1H resonances have been used as a probe for characterizing the heme electronic structure [8]. Although all 4 heme methyl ^1H resonances of the paramagnetic hemoproteins are not always resolved from the diamagnetic envelope where the signals from the apo-protein overlap severely, the observation of these resonances is essential to interpret quantitatively the in-plane asymmetry of the heme electronic structure [9]. We have not only shown that the hyperfine shifted heme methyl ^{13}C resonances of the ferric low-spin hemoproteins are resolved upfield of the ^{13}C diamagnetic envelope [10] but also demonstrated the potentiality of ^1H-^{13}C COSY in detecting the scalar connectivities between the heme methyl ^{13}C and the attached ^1H resonances, which in turn permit the assignments of all the heme methyl ^1H and ^{13}C resonances [11,12]. Therefore the heme methyl ^1H signal can be located using the ^1H-^{13}C COSY connectivity even if the signal is resonating under the apoprotein signals.

The heme methyl ^1H and ^{13}C resonances of met-cyano form of myoglobin from the shark, *Galeorhinus japonicus* (GJMbCN), have been observed, assigned, and analyzed in terms of the proximal histidyl imidazole orientation with respect to the heme. Comparison of these resonances with the corresponding resonances in met-cyano form of some hemoproteins, i.e. sperm whale Mb (SWMb), *Aplysia limacina* Mb (ALMb), horse heart cytochrome-*c* (CYT-*c*), and horseradish peroxidase (HRP), indicated that the heme methyl hyperfine shift patterns of these hemoproteins

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Abbreviations: NMR, nuclear magnetic resonance; ^1H-^{13}C COSY, ^1H-^{13}C heteronuclear shift correlated spectroscopy; Mb, myoglobin; MbCN, met-cyano form of myoglobin; SWMb, sperm whale myoglobin; ALMb, *Aplysia limacina* myoglobin; CYT-*c*, horse heart cytochrome *c*; HRP, horseradish peroxidase; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate

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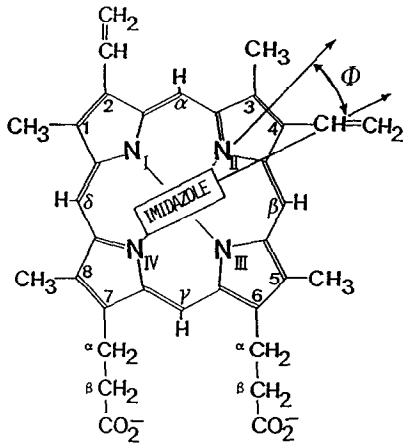

Fig. 1. Structure and numbering system for the heme. Φ is defined as the angle between the projection of the proximal histidyl imidazole plane onto the heme plane and the N_{II}-Fe-N_{IV} axis.

are quantitatively interpretable in terms of the angle (Φ) between the projection of the proximal histidyl imidazole plane onto the heme plane and the N_{II}-Fe-N_{IV} axis (see Fig. 1). The facts that the heme methyl hyperfine shift pattern of GJMbCN resembles that of SWMbCN and that its heme methyl ^1H resonances exhibit the hyperfine shifts between those of the corresponding resonances of SWMb (Φ = 19°) [13] and ALMb (Φ = 29°) [14] lead to the conclusion that the Φ-value of GJMb is slightly larger than 19°.

2. MATERIALS AND METHODS

The myoglobin of the shark, *Galeorhinus japonicus* (GJMb), was extracted from its red muscle and purified as previously described [15,16]. GJMb was oxidized by addition of 3-fold molar excess of potassium ferricyanide. The mixture was passed through a Sephadex G-50 (Sigma Chemical Co.) column equilibrated with 10 mM Bis-Tris buffer at pH 6.8 to remove ferri- or ferrocyanide, and then 5-fold molar excess of potassium cyanide was added to prepare GJMbCN. GJMbCN was concentrated to 3 mM and the solvent was exchanged to ^2H_2O in an Amicon ultrafiltration cell. The p^2H values was measured using a Toko model TP-10 pH meter with a Toko type CE103C electrode and the isotope effect was not considered to correct the p^2H value.

^1H and ^{13}C NMR spectra were obtained on a JEOL GSX-270 FT-NMR spectrometer operating at ^1H and ^{13}C frequencies of 270 and 67 MHz, respectively. A 10 mm tunable probe was used for measurements of ^{13}C and ^1H-^{13}C COSY spectra. Proton-decoupled ^{13}C NMR spectrum was recorded using 30K transients, 16K data points over 30 kHz bandwidth. ^1H-^{13}C COSY was recorded using the standard pulse sequence with $(2J)^{-1}$ = 3.6 ms. A total of 3K transients were accumulated per t_1-value with a pulse delay of 0.8 s. The initial time-domain data matrix was 2K(^{13}C-25 kHz) × 128(^1H-12 kHz) and was expanded to the final data matrix size, 2K × 256, by zero-filling. The data matrix was apodized with a sine bell function in both dimensions and the spectrum is presented in the absolute value mode. Chemical shifts are given in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for both ^1H and ^{13}C spectra.

3. RESULTS AND DISCUSSION

^1H-^{13}C COSY spectrum of GJMbCN, p^2H 7.0, at 35°C is illustrated in Fig. 2 and its ^1H and ^{13}C spectra are attached along the F_1 and F_2 axes, respectively. The ^{13}C signals resonating upfield from 0 ppm arise from either the heme methyl carbon or the heme propionate α-carbon nuclei [11,17]. The assignments of the heme 1-, 5-, and 8-CH_3 carbon resonances can be obtained from the cross-peaks with the previously assigned heme methyl proton signals [18]. As in the ^1H spectra of other MbCNs [19,20], the heme 3-CH_3 proton signal is buried inside of the diamagnetic envelope. The ^1H-^{13}C COSY connectivity is useful to locate such elusive heme 3-CH_3 proton signal and, in fact, peak D exhibits a cross-peak with a proton resonance at 6.5 ppm. Since the intensity of the cross-peaks connecting the heme propionate α-carbon and the attached proton resonances can be much smaller than those for the heme methyl resonances due to possible inequivalence of the diastereotopic propionate α-methylene proton resonances, these cross-peaks are not observed at the contour level used for Fig. 2. Therefore peak D should originate from the heme 3-CH_3 carbon and the shift of the heme 3-CH_3 proton resonance is obtained from its cross-peak. The assigned heme methyl ^{13}C resonances are compared with those of SWMb [10] and CYT-*c* [12] in Table I. The hyperfine shift pattern of the heme methyl ^{13}C resonances for GJMbCN resembles that of SWMbCN, but there exist substantial differences in the observed shifts of the corresponding resonances between the two Mbs. The smaller spread of the heme methyl resonances in GJMbCN can be simply interpreted as smaller in-plane asymmetry of the heme electronic structure because the pattern of their hyperfine shifts directly reflects the distribution of the unpaired electron density in the individual pyrrole rings [9].

There are two major factors proposed, which are possibly responsible for the in-plane asymmetry in the heme electronic structure. One is the nature and orientation, with respect to the heme, of the axial ligands [5,8,21,22] and the other is the inequivalent heme peripheral substituents whose electronic natures are also altered by interaction with the neighboring amino acids [23,24]. The former factor modulates the heme electronic structure via the interaction of P_π orbital of imidazole with the highest energy d_π orbital where the unpaired electron resides. The latter is through the molecular orbital containing the unpaired electron. Since the interaction between π-systems of the heme vinyl group and the porphyrin ring is influenced by the orientation of the vinyl group with respect to the heme plane, the electronic structure of the heme can be altered by the vinyl orientation [25]. Furthermore, the difference in the interaction between the iron-bound cyanide ion and the distal residue was also reported to perturb the hyperfine shifted heme methyl resonances

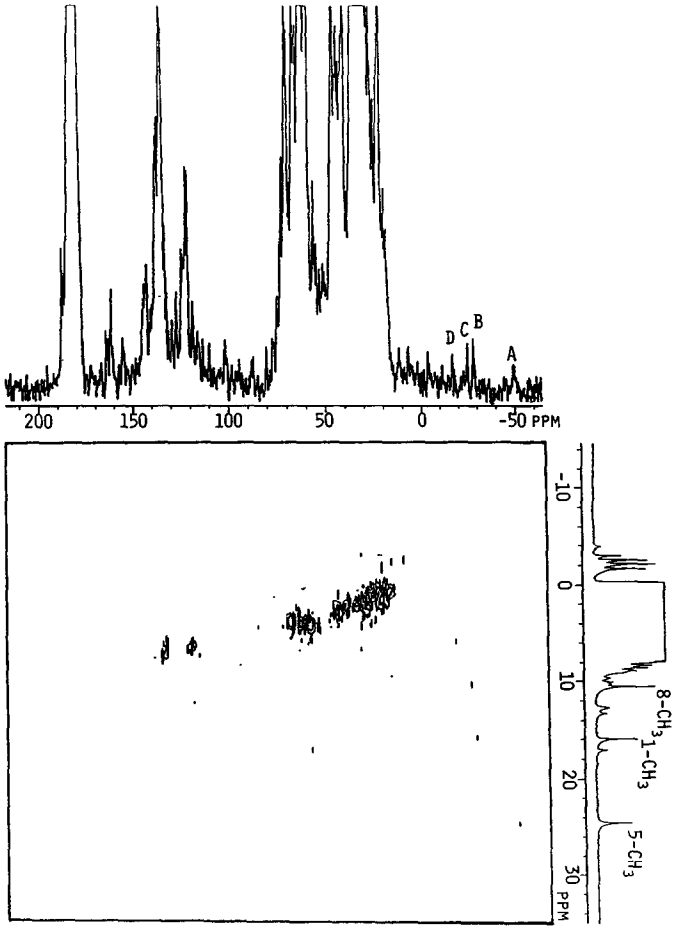

Fig. 2. ^{1}H-^{13}C COSY spectrum of GJMbCN in $^{2}H_2O$, p^{2}H 7.0, at 35°C. ^{1}H and ^{13}C spectra are shown along the F_1 and F_2 axes, respectively. The downfield hyperfine shifted heme methyl ^{1}H resonances, 1-, 5-, and 8-CH_3, were assigned previously [16].

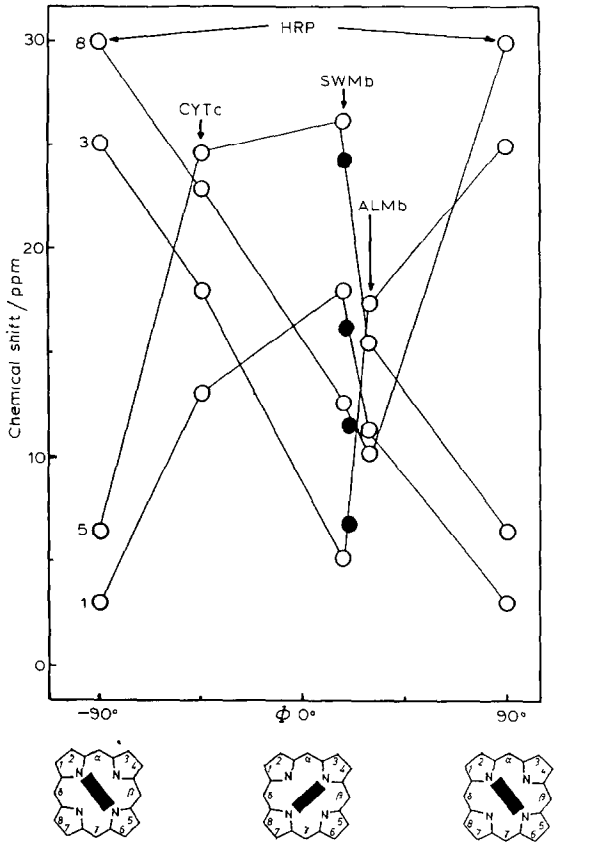

Fig. 3. Plots of the heme methyl ^{1}H hyperfine shifts of some met-cyano hemoproteins (at 35°C, p^{2}H 7.0) against Φ. The Φ values are 19° for SWMb [13], 29°C for ALMb [14], ~ −45° for CYT-*c* [28–30], and −90° for HRP [31]. The data for GJMbCN, indicated by •, fit well between those of SW and ALMbs. The results for CYT-*c* may not be compared directly with those of the other hemoproteins due to the difference in their heme peripheral side-chain groups.

[26]. But such minor perturbations on the heme electronic structure cannot account for the large difference in the hyperfine shifts for the corresponding heme methyl ^{13}C resonances between GJMbCN and SWMbCN.

Table 1

Chemical shifts[a] of heme methyl ^{13}C resonances of some met-cyano hemoproteins

Methyl carbon	GJMb	SWMb[b]	CYT-*c*[c]
1	−29.0	−35.2	−39.5
3	−17.5	−11.5	−19.3
5	−49.5	−55.5	−46.2
8	−25.8	−29.9	−50.8
Average	−30.5	−33.0	−38.9
Spread	32.0	44.0	31.5

[a] In ppm, relative to DSS at 35°C, p^{2}H 7.0

[b] Obtained from [10]. The previous assignments of the heme 3-methyl ^{1}H and ^{13}C resonances are revised and their shifts at 22°C are 5 and −13 ppm, respectively

[c] Obtained from [12]

In Fig. 3, the heme methyl ^{1}H hyperfine shifts of some hemoproteins are plotted against the angle (Φ), defined in Fig. 1. The spread of the heme methyl ^{1}H resonances which in turn reflects the asymmetric nature of the heme electronic structure is smaller for CYT-*c* and ALMb [14] and larger for SWMb [17] or HRP [27] where the imidazole plane is roughly along the N-Fe-N axis. Such Φ dependency of the in-plane asymmetry in the electronic structure of the porphyrin ring has been analyzed in detail by Soltis and Strouse [5]. The bond length between the heme iron and the bound imidazole was found to be essentially independent of the imidazole orientation in the model compounds [5] as well as hemoproteins [13,14]. Therefore it is concluded that the heme methyl hyperfine shift pattern strongly correlates with the axial imidazole orientation with respect to the heme. Therefore, although there are substantial differences in the hyperfine shifts of the corresponding heme methyl ^{1}H and ^{13}C resonances between GJMbCN and SWMbCN, the similarity in their hyperfine shift patterns between the two Mbs and the smaller spread of

the heme methyl hyperfine shifts for GJMbCN dictate that the Φ value in GJMb is slightly larger than that in SWMb. This conclusion is further supported by the fact that the hyperfine shifts of the heme methyl ^1H resonances fall in between those of the corresponding resonances of SWMbCN (Φ = 19°) [13], and ALMb (Φ = 29°) [14].

We have shown in this report that the observation of all heme methyl ^1H and ^{13}C resonances allows the interpretation of their hyperfine shift patterns in terms of the proximal histidyl imidazole orientation with respect to the heme. The determination of the proximal histidyl imidazole orientation could be of great use for interpreting the relationship between the functional properties of hemoproteins and their active site structures.

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