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Assignment of hyperfine shifted haem methyl carbon resonances in paramagnetic low-spin met-cyano complex of sperm whale myoglobin

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The hyperfine shifted resonances arising from all four individual haem carbons of the paramagnetic low-spin met-cyano complex of sperm whale myoglobin have been clearly identified and assigned for the first time with the aid of ^{1}H-^{13}C heteronuclear chemical shift correlated spectroscopy. Alteration of the in-plane symmetry of the electronic structure of haem induced by the ligation of proximal histidyl imidazole spreads the haem carbon resonances to 32 ppm at 22°C, indicating the sensitivity of those resonances to the haem electronic/molecular structure. Those resonances are potentially powerful probes in characterizing the nature of haem electronic structure.

^{13}C-NMR; Myoglobin; Hyperfine shift; Heme; (Sperm whale)

1. INTRODUCTION

NMR of paramagnetic haemoproteins has provided a wealth of information on the electronic/molecular structure of their prosthetic haem groups because the observed hyperfine shifted NMR signals arising from the haem and the amino acid residues oriented in the close proximity to the haem iron can be interpreted quantitatively in terms of the interaction between the nucleus and the unpaired electron(s) of the haem iron [1–5]. To date, those data have been obtained primarily from ^{1}H NMR studies and relatively little ^{13}C NMR work has been reported on paramagnetic haemoproteins due to the inherent low NMR sensitivity of ^{13}C nucleus. Potential usefulness of carbon hyperfine shift data in characterization of haem electronic structure has been demonstrated in the studies of paramagnetic model compounds [6–15]. Comparison of haem proton and carbon shifts may permit clear separation of relative contributions to their hyperfine shifts and therefore provides quantitative characterization of the π electron density distribution pattern within the haem [7,12,15].

Natural-abundance ^{13}C NMR spectra have been reported for met-cyano complex of various cytochrome *c* and myoglobins [16–19]. But, in earlier studies, hyperfine shifted carbon resonances arising from the haem have not been clearly identified. We have reexamined the natural-abundance ^{13}C NMR spectrum of met-cyano sperm whale myoglobin and the hyperfine shifted haem carbon resonances have been clearly observed in the spectrum. From the comparison of the spectrum of metMbCN with that of the model compound, paramagnetic low-spin protoporphyrin IX dicyano complex, the hyperfine shifted haem carbon resonances have been identified and

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Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulphonate; ^{1}H-^{13}C COSY, ^{1}H-^{13}C heteronuclear chemical shift correlated spectroscopy; Mb, myoglobin; metMbCN, met-cyano myoglobin

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the individual haem methyl carbon resonances have been unambiguously assigned using the ^{1}H-^{13}C COSY connectivities. We, therefore, report herein on the assignments of some hyperfine shifted haem carbon resonances including all four individual haem methyl carbon resonances in metMbCN which are extremely valuable probes for the characterization of the electronic structure of haem by ^{13}C-NMR.

2. MATERIALS AND METHODS

Sperm whale myoglobin (type II) and protohemin IX were purchased from Sigma. The paramagnetic low-spin met-cyano complex of myoglobin was prepared in $^{2}H_2O$ (8 mM) with a 5-fold excess of potassium cyanide (KCN) and with a p^{2}H value adjusted to 7.5 using 0.2 M $NaO^{2}H$ and ^{2}HCl. Any precipitate was removed by centrifugation. The paramagnetic low-spin dicyano complex of protohemin IX was prepared in $C^{2}H_3O^{2}H$ (20 mM) with a large excess of KCN.

Proton-decoupled 67.8 MHz ^{13}C NMR spectra were recorded on a Jeol GX-270 FT-NMR spectrometer equipped with a 10 mm tunable probe, utilizing 20K transients with 16K data points over 30 kHz bandwidth. The ^{13}C spectra were apodized with an exponential window function which introduced 10 Hz linebroadening. ^{1}H-^{13}C COSY was obtained using the standard pulse sequence [20] with $(2J)^{-1} = 3.6$ ms. A total of 1K transients were accumulated per t_1 value with a pulse delay of 1 s. The initial data matrix was 2K(^{13}C-22 kHz) × 64(^{1}H-14 kHz) in ω_2 and ω_1 dimensions, respectively, and was expanded to the final data matrix size 2K × 256 by zerofilling. The data matrix was apodized with an exponential function in both dimensions and the absolute value mode is presented. Chemical shifts are given in ppm downfield from DSS.

3. RESULTS AND DISCUSSION

The 67.8 MHz ^{13}C NMR spectrum of the paramagnetic low-spin metMbCN in $^{2}H_2O$, p^{2}H 7.5, at 22°C is illustrated in trace A of fig.1. Compared with the spectrum of the diamagnetic carbonmonoxy Mb reported by Oldfield et al. [17], numerous ^{13}C signals are newly resolved in the regions of −65–10, 70–105, 145–155 and 185–190 ppm of the metMbCN spectrum. From their unusual shifts and linewidths (~100 Hz), it is apparent that they are hyperfine shifted resonances arising from ^{13}C nuclei which interact with the unpaired electron(s) of haem iron through either a through-space dipolar interaction or delocalization of the spin into an orbital centred on the nucleus. The spectrum of the paramagnetic low-spin iron(III) protoporphyrin IX dicyano complex in $C^{2}H_3O^{2}H$ at 22°C is shown in trace B for comparison and the signal assignments [21] are given with the spectrum. Although hyperfine shifted haem resonances exhibit solvent dependency in their shifts [2], the hyperfine shifted ^{13}C resonances observed in both spectra can be compared qualitatively. Hyperfine shifted haem methyl ^{13}C resonances are identified as the most upfield shifted signals (see trace B), therefore upfield hyperfine shifted resonances, a–d, are most likely to be the haem methyl carbon resonances of metMbCN. The individual haem methyl carbon assignments are performed using ^{1}H-^{13}C COSY connectivities (see below). Recently Sankar et al. [22] have reported the assignments of the vinyl carbon resonances in the spectra of various complexes of Mb obtained by using the reconstituted Mb with specifically ^{13}C labelled haems at α or β carbons of haem vinyl groups [23]. According to their results, the signals from 2-C_α, 4-C_α, 2-C_β and 4-C_β in metMbCN resonate at 51.3, 81.2, 186.7, and 147 ppm, respectively, at 25°C. The resonance of 2-C_α appears to be under the protein resonances. But three other resonances can be clearly observed in trace A. Although the other hyperfine shifted resonances cannot be assigned at present, we confidently assign the resonance at 189.4 ppm to the carboxylate carbon of the haem because it not only exhibits the similar shift to the corresponding resonances in the spectrum of trace B but also has a relatively narrow linewidth.

The ^{1}H-^{13}C COSY spectrum of metMbCN, together with one-dimensional ^{1}H and ^{13}C NMR spectra, are illustrated in fig.2. In the ^{1}H spectrum, the downfield hyperfine shifted haem methyl proton resonances have been assigned using isotopically labelled haems [24]. In the contour spectrum, the cross-peaks connecting the particular ^{1}H and ^{13}C resonances immediately reveal the assignments of some ^{13}C resonances. The cross-peaks connecting upfield shifted carbon

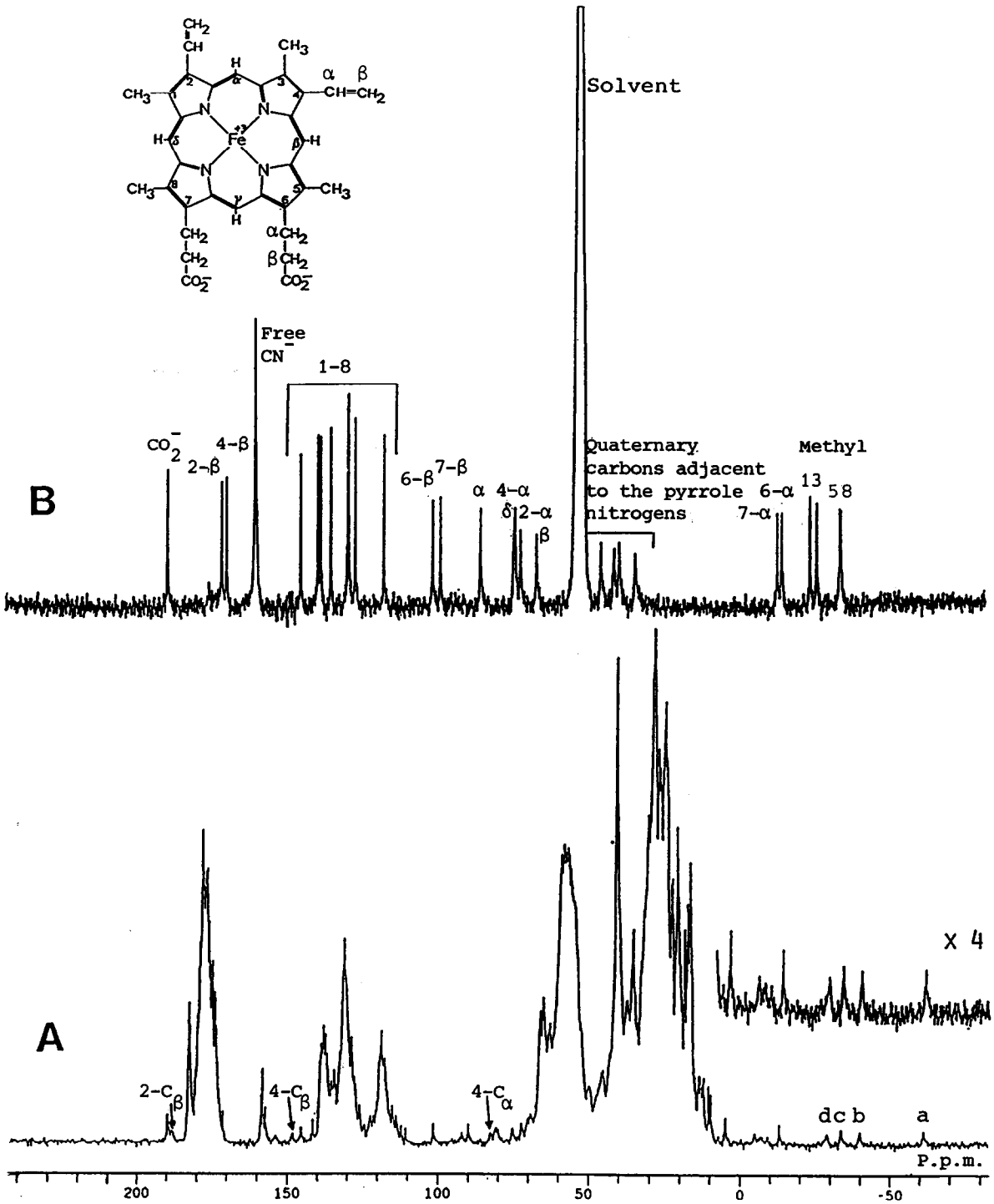

Fig.1. The 67.8 MHz proton-decoupled ^{13}C-NMR spectra of metMbCN in $^{2}H_2O$, p^{2}H 7.5, at 22°C (A) and the paramagnetic low-spin iron(III) protoporphyrin IX dicyano complex in $C^{2}H_3O^{2}H$ at 22°C (B). The structure and numbering system of the haem are given in the inset. The resonance assignments [21] of haem dicyano complex are given in B. The vinyl carbon resonances recently assigned by Sankar et al. [22] are indicated by arrows in A.

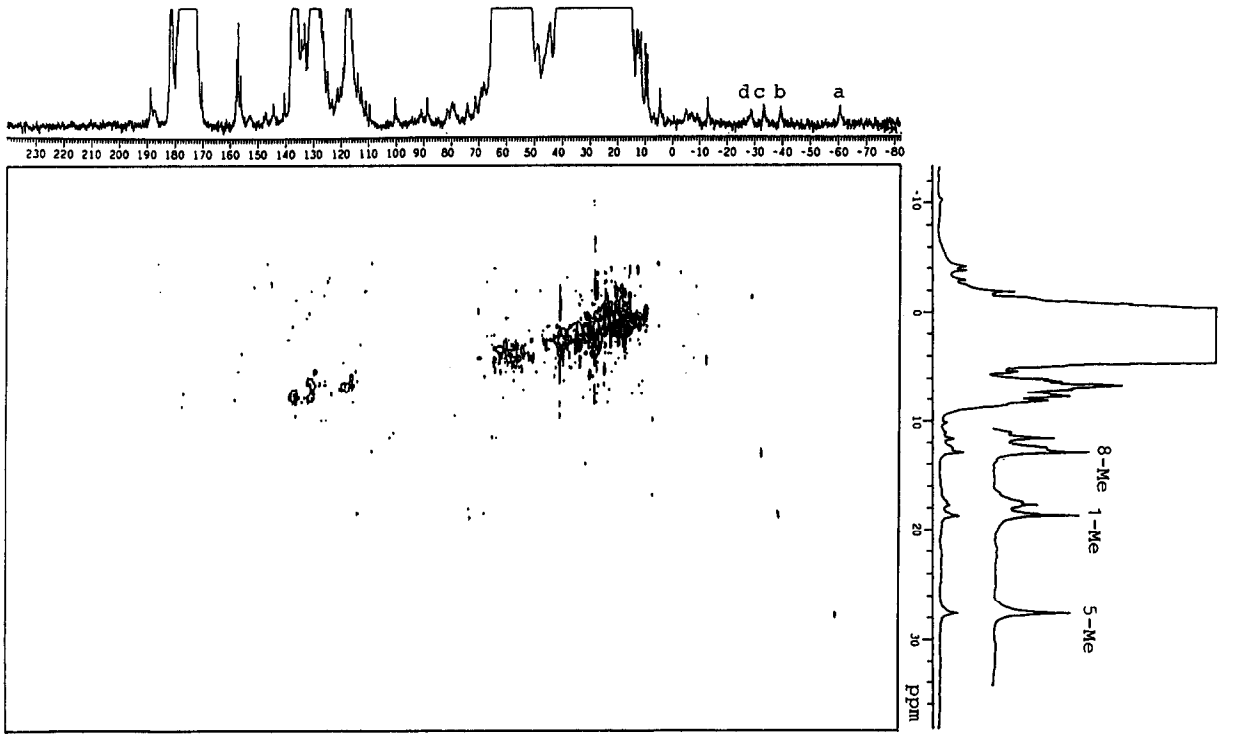

Fig.2. ^{1}H-^{13}C COSY spectrum of metMbCN in $^{2}H_2O$, p^{2}H 7.5, at 22°C. One-dimensional ^{1}H and ^{13}C spectra are shown along the F_1 and F_2 axes, respectively. The assignment of downfield hyperfine shifted haem methyl proton resonances has been carried out by La Mar et al. [24] using specifically deuterated haems. The ^{1}H-^{13}C COSY connectivities revealed the assignment of haem methyl carbon resonances.

resonances at −60.5, −39.3, and −33.2 ppm to 5-, 1- and 8-methyl proton resonances clearly indicate the assignments of hyperfine shifted carbon signals a, b, and c to 5-, 1- and 8-methyl carbons, respectively. Furthermore, as discussed above, the ^{13}C resonance at −28.5 ppm exhibits not only significant upfield shift which is characteristic of haem methyl carbon resonances, but also comparable linewidth with those of the haem methyl carbon resonances, strongly suggesting the assignment of resonance d to the remaining haem methyl carbon, i.e., 3-methyl carbon. Resonance d shows a cross-peak to a ^{1}H signal resonating at −1.7 ppm, identifying the 3-methyl proton resonance which was not assigned previously. Hyperfine shifts of haem methyl proton and carbon and carboxylate carbon resonances for iron(III) protoporphyrin IX dicyano complex and metMbCN are compared in table 1. Although they are different in the direction of shift, a parallel relationship in the magnitude of hyperfine shift between carbon and attached proton resonances are observed, reflecting dominant π-type contact shift contribution to haem methyl resonances. The ligation of proximal histidyl imidazole alters the in-plane symmetry of the electronic structure of haem and spreads the haem carbon resonances to 32 ppm, indicating the sensitivity of those resonances to the electronic structure of haem. The 3-methyl proton resonance, which was assigned in this study, exhibits unusual upfield hyperfine shift relative to the diamagnetic haem methyl proton shift of 3.5 ppm [25] whereas the 3-methyl carbon resonance shifts in the same direction as those of the other three methyl carbons. More detailed information is needed to explain the anomaly of the 3-methyl proton shift. Restricted methyl rotation due to contact with the surrounding amino acid residues may contribute to interference of unpaired spin delocalization via hyperconjugation to the protons of this methyl group and therefore interaction of haem methyl groups with the sur-

Table 1

Haem methyl ^{1}H and ^{13}C and carboxylate ^{13}C chemical shifts in iron(III) protoporphyrin IX dicyano complex and metMbCN (in ppm from DSS at 22°C)

	Protoporphyrin IX[a]		MetMbCN[b]	
	^{1}H shift	^{13}C shift	^{1}H shift	^{13}C shift
Haem methyl				
1	13.26	−26.5	18.77	−39.3
3	14.35	−28.7	−1.7	−28.5
5	17.52	−36.2	27.63	−60.5
8	17.96	−36.8	12.97	−33.2
Average shift[c]	12.27	−32.1	12.64	−40.4
Spread	4.70	10.3	29.3	32.0
Carboxylate		184.8		189.4

[a] In $C^{2}H_{3}O^{2}H$

[b] In $^{2}H_{2}O$

[c] Since the diamagnetic reference shift for the haem methyl proton is 3.5 ppm [25], the quantity,

$\frac{1}{4}(\sum_{i=1,3,5,8}|\delta_{i\text{-Me}} - 3.5|)$

is indicated for the ^{1}H shift data

rounding protein may be included to interpret the large spread of haem methyl proton resonances in metMbCN. In the ^{1}H-^{13}C COSY spectrum, the ^{13}C signal at 189.4 ppm does not exhibit a cross-peak, supporting the assignment of this resonance to the carboxylate carbon.

The similar hyperfine shifted haem carbon resonances should be observable in the natural-abundance ^{13}C NMR spectra of low-spin complexes of various haemoproteins and ^{1}H-^{13}C COSY connectivities can be quite effectively utilized to assign those resonances from the known ^{1}H assignment. Such studies are in progress and the results will be published elsewhere.

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