Assignment of hyperfine-shifted heme carbon resonances in ferricytochrome b_5

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Abstract The reverse detection heteronuclear multiple quantum coherence, HMQC, study of native bovine ferricytochrome b_5 has provided the complete assignment of hyperfine shifted resonances of heme carbons attached proton(s). The dominant delocalized π-spin density to vinyl groups gives rise to contact shifts which have opposite direction for a carbon and its attached proton(s). The most hyperfine shifted ^{13}C heme signals are mainly generated from 3rd heme pyrrole ring substituents which identifies that the molecular orbital for facile electron transfer is oriented to exposed heme edge. Magnetic/electronic asymmetry of heme induced by two axial His makes spread the hyperfine shifted heme carbon resonances over the range of 280 ppm at 25°C, which would be the more sensitive probe than those of proton resonances in characterizing the nature of heme electronic structure of ferricytochrome b_5.

Key words: Cytochrome b_5; Heme resonances; Hyperfine shift; ^{13}C NMR

1. Introduction

Ferricytochrome b_5 is a microsomal membrane-bound heme protein of 12 kDa involved in fatty acid desaturation [1], the cytochrome P_{450} reductase system [2] and the hemoglobin reductase system [3,4]. These biologically important implications of cytochrome b_5 have led to the subject of the intensive NMR studies for many years [5–17]. In particular, since the structural feature of heme active site modulates redox potential and electron transfer to the redox partner proteins [14,15,18,19], it is crucial to understand NMR parameters of the resonances from heme pocket in terms of the structural and functional relationship.

NMR of ferricytochrome b_5 has provided a wealth of information on the electronic/magnetic structure of heme groups and nearby amino acid residues because the observed hyperfine shifted NMR signals arising from the heme side chains and amino acid residues oriented in the close proximity to the heme iron can be interpreted quantitatively in terms of the interaction between the nucleus and the unpaired electrons of the heme iron [12]. To date, those data have been obtained only from ^1H NMR studies using specifically deuterated heme and nuclear Overhauser effect experiments [5–12] and any ^{13}C NMR investigation has not been reported on cytochrome b_5 due to the inherent low sensitivity of ^{13}C nucleus and limited amount of protein isolated from bovine liver. In spite of poor sensitivity of natural abundance ^{13}C NMR, if we are able to assign the paramagnetic heme ^{13}C signals, it may be able to provide some insight into the origin of ^{13}C chemical shifts due to the electronic asymmetry of heme iron [20]. Also, it assists the cross-assignment of resonances in the less resolved signal assignment by ^1H nuclear Overhauser effect due to the much larger chemical shift dispersion. Finally, determining the contact and pseudocontact contribution to the hyperfine ^{13}C chemical shift would allow to estimate the unpaired electron spin density distribution in heme and axial ligands.

Natural abundance ^{13}C NMR spectra have been reported for cytochrome *c* [21,22] and metcyano-myoglobins [23,24]. They have been assigned using specifically ^{13}C-enriched heme reconstituted protein, DEPT and conventional ^1H-^{13}C COSY which has much less sensitivity than HMQC. However, earlier studies, hyperfine-shifted carbon resonances arising from the heme have not been fully identified. In spite of a substantial sensitivity advantage of HMQC [25,26] over the conventional shift-correlation experiment, it, however, has not yet gained the popularity one might expect on the basis of intrinsic advantages. Less attention has been directed toward HMQC for ^{13}C resonance assignments of paramagnetic heme proteins. This neglect is likely due to the belief that the severe line broadening that can accompany paramagnetism obscures the ^1H-^{13}C connectivities and renders the detection of coherence in a HMQC map experimentally impossible.

We report on the full assignments of hyperfine shifted heme carbons attached with proton(s) in bovine ferricytochrome b_5 for the first time using HMQC experiment which detects the characteristic ^1H-^{13}C scalar connectivity of paramagnetic heme, and those are extremely valuable probes for the characterization of the heme electronic structure by ^{13}C NMR.

2. Materials and methods

Cytochrome b_5 was isolated from fresh bovine liver as described previously [18]. An NMR sample consists of 8 mM solution of ferricytochrome b_5 in ^2H_2O. The pH was adjusted as required by the addition of small amounts of 0.2 M ^2HCl or 0.2 M NaO^2H and was measured using a Beckman model Φ34 pH meter equipped with an Ingold microcombination electrode; pH values were not corrected for the isotope effect.

Proton detected heteronuclear multiple quantum coherence (HMQC) was acquired on a Varian Unity *plus 600* equipped with a two-channel NMR interface and a double resonance $^1H/^{13}C$ 5 mm indirect probe. All protons not coupled to ^{13}C were inverted by the bilinear (BIRD) pulse in spite of the lower sensitivity by the negative NOE effect.

The spectral width and number of points acquired were 4000 Hz and 4096 complex points in ^1H(F2), 50000 Hz and 256 real points in ^{13}C(F1). The initial data matrix was expanded to 4096 × 1024 by linear prediction, then zero-filled to 4096 × 2048. Broad band decoupling with the GARP sequence was used during the acquisition period. 512 transients were accumulated per increment. The total acquisition time was ~10 h. Solvent suppression was achieved by on resonance presaturation of the solvent signal.

The spectrum was acquired at 298 K and was recorded with States-Haberkorn method [27] for the non-acquired dimension. Spectrum was

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processed with NMR software (version 4.3) provided by Varian Associate. The data matrix was apodized with Gaussian function in both dimensions. ^{1}H chemical shifts are given in ppm from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). ^{13}C chemical shifts were measured indirectly by multiplying the ^{1}H frequency of the HDO resonance with 0.25145002 (the $^{13}C/^{1}H$ frequency ratio in TMS) [26].

3. Results and discussion

The highly resolved downfield-shifted region (^{13}C dimension) of HMQC spectrum for ferricytochrome b_5 is shown in Fig. 1. HMQC spectrum demonstrates the ability to map covalent connectivities between heme carbon and attached protons with a much higher sensitivity advantage over the conventional ^{1}H-^{13}C COSY experiment. Heme carbons attached with proton(s) were assigned unambiguously on the basis of previously assigned hyperfine-shifted heme proton resonances [12]. The cross-peak notations were followed by those of heme proton resonances published earlier [12]. Resonances, Y and Z which correspond to 2-$V_{\beta c\&t}$ in ^{1}H(F2) dimension, give the covalent connectivity between 2-V_β protons and carbon (222.3 ppm) in ^{13}C(F1) dimension. Cross peaks, R and Z' which are 6-propionate β-protons, show a 6-propionate β-carbon chemical shifts (140.3 ppm). Cross-peak, O' which was assigned to 4-$V_{\beta t}$ by strong NOE from 3-CH_3 heme group in ^{1}H NOESY map, indicates 4-V_β carbon chemical shift. In the earlier paper describing about heme proton assignments, 4-$V_{\beta c}$ proton was not clearly identified from NOESY and COSY map due to ambiguity in diamagnetic region. It was tentatively assigned that 4-$V_{\beta c}$ proton would be degenerate with 4-$V_{\beta t}$ proton [12]. However, 4-$V_{\beta c}$ proton can be assigned unambiguously from the same connectivity with 4-V_β carbon (127.0 ppm), which was labelled O". It was speculated that the vinyl orientations can influence redox potential of cytochrome b_5; furthermore, the electron withdrawing properties of heme vinyl groups are modulated by the variable degree of coplanarity of vinyls and heme π system [18]. For the two vinyl groups, we had shown that observed NOE data are consistent with a sterically clamped, largely in-plane 4-vinyl group restricted with neighboring protein matrix. Therefore, from the our unambiguous assignment of 4-$V_{\beta c}$ proton we are able to find out that there is no NOE between 3-CH_3 and 4-$V_{\beta c}$, which indicates that 4-vinyl group is not oriented to the 3-CH_3 group. This fact confirmed that 4-vinyl group is restricted with neighboring protein matrix. Cross-peaks, X and R' assigned to 7-propionate β-protons in F2 dimension, correspond to 7-propionate β-carbon (105.4 ppm). Cross-peak, V assigned to α-meso-proton in ^{1}H dimension, shows a α-meso carbon chemical shift (57.6 ppm). Cross-peak, H' assigned to 4-V_α proton in ^{1}H dimension, shows a 4-V_α carbon chemical shift (59.0 ppm). This assignment was confirmed by the temperature sensitivity (not shown).

The highly resolved upfield-shifted region (^{13}C dimension) of HMQC spectrum for ferricytochrome b_5 is shown in Fig. 2.

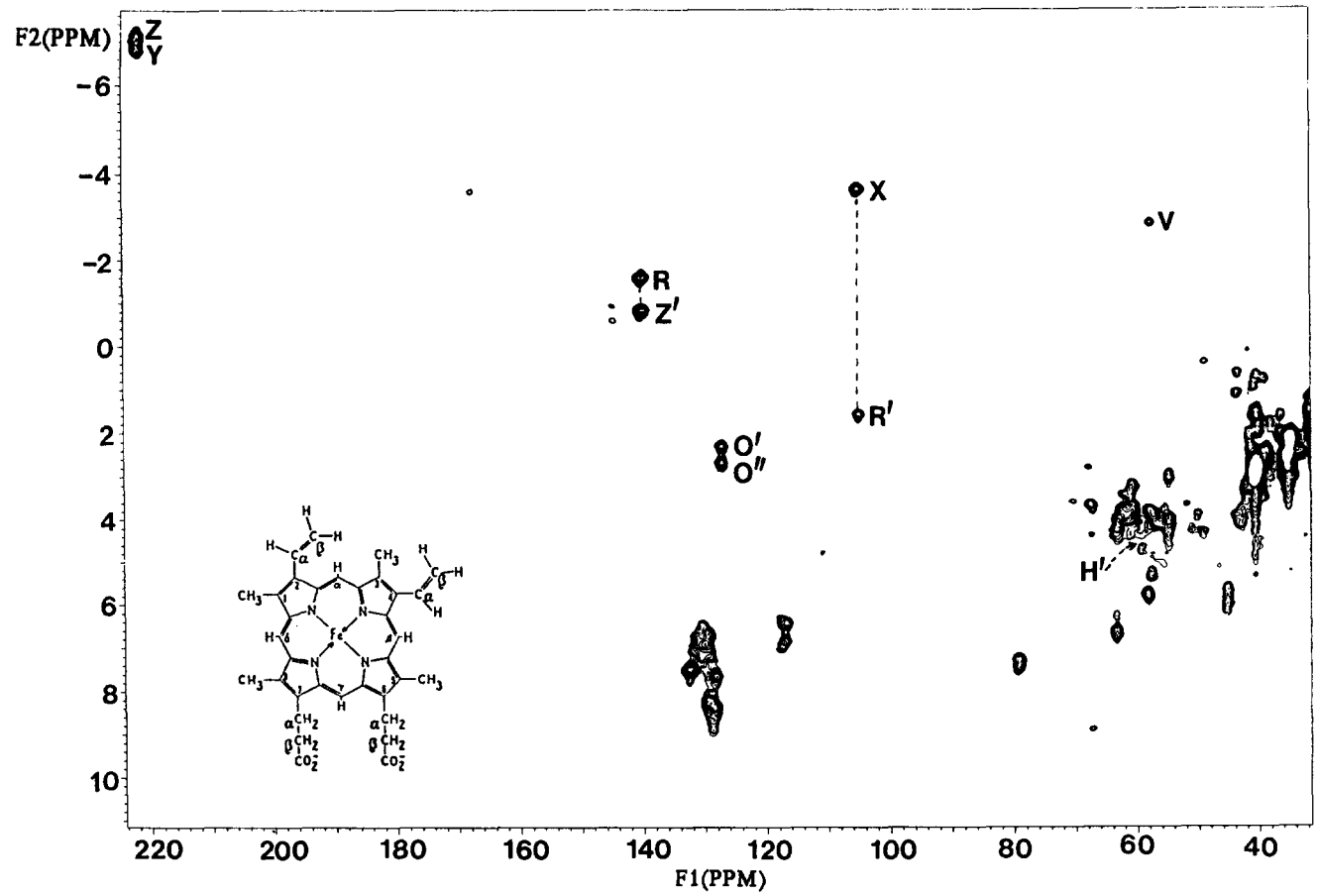

Fig. 1. A region of 600-MHz (^{1}H frequency) HMQC spectrum of bovine ferricytochrome b_5, pH 6.5, 25°C, showing ^{1}H-^{13}C coherence between 30 and 225 ppm (^{13}C dimension) window and −8 to 11 ppm (^{1}H dimension) region. The spectrum results from 4096 × 2048 data matrix. The structure and numbering system of the heme are given in the inset.

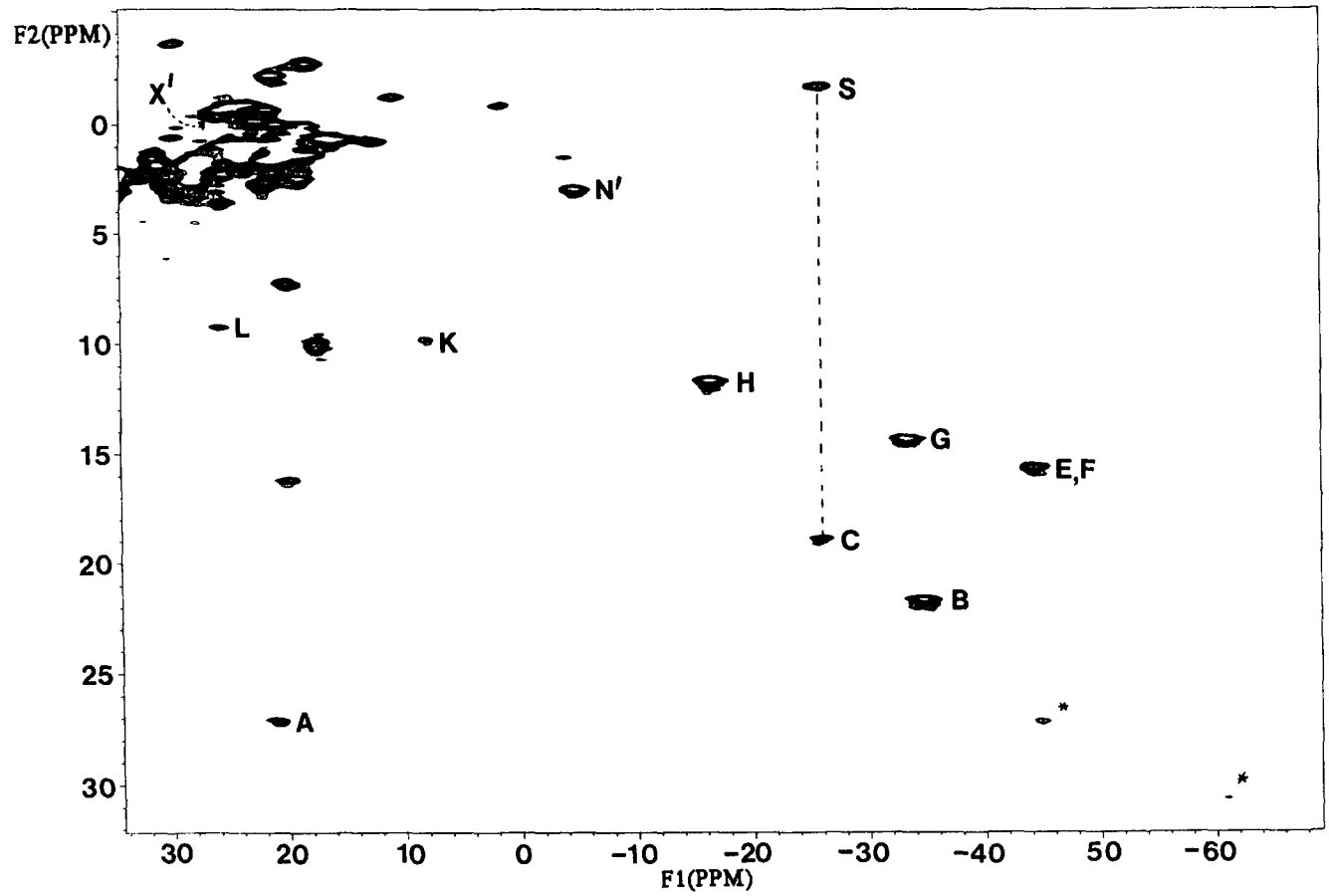

Fig. 2. A region of 600-MHz (^{1}H frequency) HMQC spectrum of bovine ferricytochrome b_5, pH 6.5, 25°C, showing ^{1}H-^{13}C coherence between −70 and 35 ppm (^{13}C dimension) window and −5 to 32 ppm (^{1}H dimension) region. The spectrum results from 4096 × 2048 data matrix.

Cross-peak A assigned to 2-V_α proton in ^{1}H dimension, shows a 2-V_α carbon chemical shift (20.6 ppm). As ^{1}H NMR studies showed that the major fraction of the conformationally heterogeneous protein has the heme rotated 180° about the $\alpha\gamma$-meso axis with respect to that characterized in the initial X-ray data [12], the cross-peaks labelled by * are due to signals from reverse oriented heme. Cross-peak B assigned to 5-CH_3 protons in ^{1}H dimension, shows a 5-CH_3 carbon chemical shift (–34.8 ppm). Cross-peaks C and S which were assigned to 7-propionate α- protons in F2 dimension, correspond to 7-propionate α-carbon (–26.2 ppm) in F1 dimension. Cross-peaks E and F which were assigned to 6-propionate α-protons in F2 dimension, correspond to 6-propionate α-carbon (–44.5 ppm) in F1 dimension. These strong hyperfine-shifts for 6-propionate and 2-vinyl carbons indicate that the molecular orbital for facile electron transfer is oriented to align between 2- and 6-positions of the heme (the inset of Fig. 1). Cross-peak G assigned to 3-CH_3 protons in ^{1}H dimension indicates a 3-CH_3 carbon chemical shift (–33.3 ppm). Cross-peak K assigned to δ-meso protons in ^{1}H dimension, indicates a δ-meso carbon chemical shift (7.8 ppm). Cross-peak L assigned to β-meso protons in ^{1}H dimension, indicates a β-meso carbon chemical shift (25.4 ppm). Although δ- and β-mesos are almost degenerate in the ^{1}H chemical shift, the chemical shift differnence in ^{13}C dimension is about 17 ppm. Cross-peak N' assigned to 8-CH_3 protons in ^{1}H dimension, indicates a 8-CH_3 carbon chemical shift

Table 1
Assignment of ^{13}C chemical shifts of heme carbon resonances in bovine ferricytochrome b_5 at 25°C

Label	Chemical shift (^{1}H)[a]	Chemical shift (^{13}C)[b]	Assignment
A	27.43	20.6	2-V_α
B	21.84	−34.8	5-CH_3
C	18.97	−26.2	7-P_α
S	−1.81		7-P_α'
E	15.76	−44.5	6-P_α
F	15.76		6-P_α'
G	14.40	−33.3	3-CH_3
H	11.45	−16.6	1-CH_3
K	9.79	7.8	δ-meso
L	9.20	25.4	β-meso
H'	4.80	59.0	4-V_α
N'	2.70	−4.6	8-CH_3
O'	2.35	127.0	4-$V_{\beta t}$
O''	2.64		4-$V_{\beta c}$
R'	1.57	105.4	7-P_β
X	−3.52		7-P_β'
X'	−0.27	26.5	γ-meso
Z'	−0.82	140.3	6-P_β
R	−1.56		6-P_β'
V	−2.89	57.6	α-meso
Y	−6.75	222.3	2-$V_{\beta c}$
Z	−7.04		2-$V_{\beta t}$

[a]Ref [12]. [b]This study.

(−4.6 ppm). Cross-peak X' assigned to γ-meso protons in ^{1}H dimension, indicates a γ-meso carbon chemical shift (26.5 ppm). As described above all heme carbon resonances attached with protons were assigned unambiguously and were tabulated in Table 1. A parallel relationship in the magnitude of hyperfine shift between carbons and attached protons are generally observed despite of opposite direction of shifts. A detailed attempt to separate relative contributions to the paramagnetic carbon shifts should await the unambiguous assignment of heme pocket amino acid residue assignment. The ^{13}C resonances assignment of paramagnetic amino acid residues located in heme pocket are currently being explored and the results will be published elsewhere. Also, the similar hyperfine shifted heme carbon resonances should be observable in the natural abundance ^{13}C HMQC spectra of low-spin heme proteins (e.g. myoglobins, cytochromes) and can be quite effectively utilized to assign those resonances from the known ^{1}H assignment. Such studies are currently under progress.

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