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Determination of the functionally important heme peripheral vinyl group orientation in paramagnetic hemoprotein by 2D NMR

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2D NMR spectroscopies have been successfully used to characterize the heme peripheral vinyl groups in paramagnetic hemoprotein in spite of the difficulties from the rapid paramagnetic relaxation and the low digital resolution of the 2D NMR map. The scalar coupling network system among the vinyl protons is clearly identified in the COSY spectra from its characteristic cross-peak pattern and the dipolar coupling connectivities of the vinyl proton resonances with other heme side-chain proton resonances not only provide the specific assignment of vinyl β-proton resonances but also allow the determination of the vinyl group orientation with respect to the heme plane.

NMR, 2D; Heme; Myoglobin; Resonance assignment; Vinyl group orientation

1. INTRODUCTION

The functional properties of hemoproteins depend crucially on the heme peripheral substituent groups [1]. Especially, the role of the ubiquitous vinyl groups in the functions of b-type hemoproteins has been studied in terms of not only the electronic nature [1] but also the orientation of the heme vinyl groups with respect to the heme plane [2–6]. The interaction of the heme vinyl group with a nearby amino acid residue in the active site of human adult hemoglobin has been proposed to play an important role in the initial steps of its cooperative ligand binding process [7]. The orientation of the vinyl substituents with respect to the heme plane was found to modulate their electron-withdrawing ability and hence the reduction potential of the heme center in cytochrome b_5 [2] and cytochrome-*c* peroxidase [3] is influenced.

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Although the information about the heme vinyl group orientation in a variety of hemoproteins has been usually obtained from the X-ray structural analysis [4–7], it has been shown that solution NMR studies on paramagnetic hemoproteins can be used to determine the orientation of the heme vinyl substituents with respect to the heme plane [8–10] and the dynamic nature of the vinyl groups in hemoproteins [9,10].

We present herein the results of 2D NMR studies of a paramagnetic low-spin myoglobin which uniquely determine the orientation of the heme peripheral vinyl group with respect to the heme plane. The spin coupling network system among the vinyl proton resonances is clearly identified from a characteristic cross-peak pattern in the ^{1}H-^{1}H COSY spectrum and the specific assignment of vinyl β-proton resonances is obtained with the aid of NOESY connectivities. The dipolar coupling connectivities of the vinyl proton resonances with the other heme peripheral side-chain proton resonances, observed as the cross-peaks in NOESY spectrum, are interpretable in terms of the vinyl group orientation with respect to the heme plane.

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2. MATERIALS AND METHODS

The myoglobin from the shark, *Galeorhinus japonicus*, was isolated and purified as previously reported [11,12]. The *Galeorhinus japonicus* myoglobin (GJMb) was then oxidized by the addition of 3-fold molar excess of potassium ferricyanide and 5-fold molar excess of KCN was added to met-GJMb to prepare met-cyano GJMb (GJMbCN). The sample was concentrated to ~1 mM and then the solvent was exchanged with ^2H_2O in an Amicon ultrafiltration cell. p^{2}H of the sample was adjusted to ~8.5.

^{1}H NMR spectra were obtained on a Jeol GSX-270 FT-NMR spectrometer. 1D spectrum consisted of ~3000 transients with 8K data points over 15 kHz spectral width using 90° pulse of 8 μs. The residual water signal was suppressed by a decoupler pulse. 2D spectra were recorded with the standard pulse sequences [13]. The 256 free induction decays were acquired with 1K data points and a spectral width of 10 kHz. The time-domain data matrix was expanded to 512 × 512 by zero-filling. A sine bell function was used to apodize the spectrum in both dimensions and the spectrum is presented in the absolute value mode. The NOESY spectrum was obtained on a Jeol GSX-500 FT-NMR spectrometer and the mixing time of 100 ms was used to avoid problems of spin diffusion [14]. Chemical shifts are given in ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

3. RESULTS AND DISCUSSION

^{1}H-^{1}H COSY spectrum of GJMbCN, p^{2}H 8.5, at 52.5°C, together with the ^{1}H spectrum, is illustrated in fig.1. Although the distal histidine residue is replaced by a glutamine residue in GJMb [12], ^{1}H NMR spectral properties of GJMb resemble those of the met-cyano form of the previously well-studied common myoglobins such as sperm whale skeletal muscle and horse heart myoglobins. Three heme methyl proton signals, A–C, and four signals with single-proton intensity, a–d, are resolved below ~10 ppm. Their Curie plots, the observed shifts versus reciprocal of the absolute temperature, are given in fig.2. Since the met-cyano complex of hemoproteins is essentially in pure low-spin state, the plots follow Curie's law. The curvature of the plot for peak a, however, is apparent and its characteristic deviation from Curie's law [15] as well as its shift value [16–18] strongly support the assignment of this signal to the vinyl α-proton resonance. This assignment is confirmed in the COSY spectrum of fig.1. In the 2D contour map, peak a exhibits the scalar coupling connectivities with the upfield hyperfine shifted resonances at −1.78 and −2.20 ppm which exhibit doublet structures. Upfield hyperfine shifted vinyl β-proton resonances have been

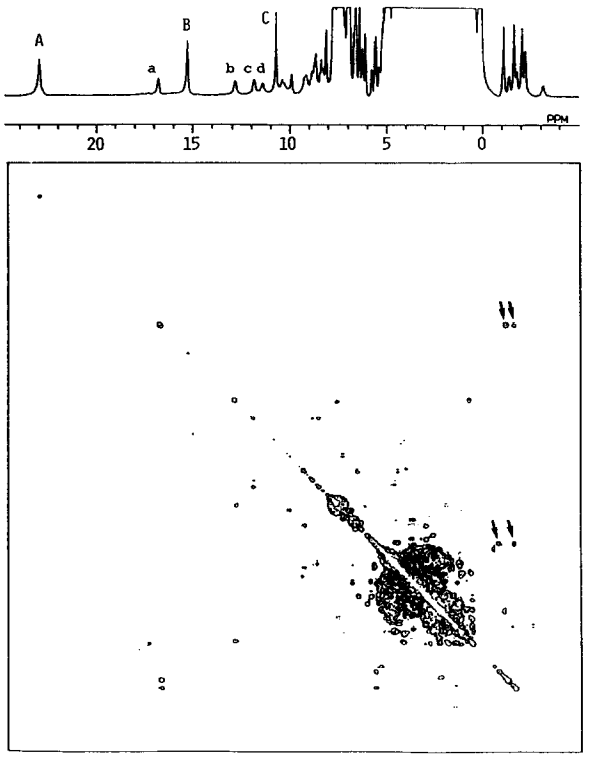

Fig.1. ^{1}H-^{1}H COSY spectrum of GJMbCN, p^{2}H 8.5, at 52.5°C. ^{1}H 1D spectrum of GJMbCN is attached. The scalar coupling connectivities between the α- and β-proton resonances of 2- and 4-vinyl groups are clearly identified from its characteristic cross-peak pattern as indicated by arrows in the COSY map. the cross peak for the geminal pair of the vinyl β-protons appears to be varied under the diagonal peaks. Peaks A, B and C are assigned to heme 5-, 1-, and 8-CH_3 resonances, respectively (see text).

reported for various paramagnetic low-spin hemoproteins [9,10,19–22] and model compounds [18]. Therefore the resonances at −1.78 and −2.20 ppm are assigned to the vinyl β-protons associated with the vinyl α-proton resonating at 16.75 ppm. Other two upfield hyperfine shifted doublet resonances at −1.39 and −2.20 ppm also exhibit cross-peaks to an identical resonance at 5.4 ppm and they are likely to originate from the protons of the other heme peripheral vinyl group. Although the scalar coupling constants between the vinyl α-proton and β-protons are often masked from the structure of the vinyl α-proton resonance by its broad linewidth (~40 Hz) due to the effective paramagnetic relaxation, the spin coupling networks between them can be clearly reflected in

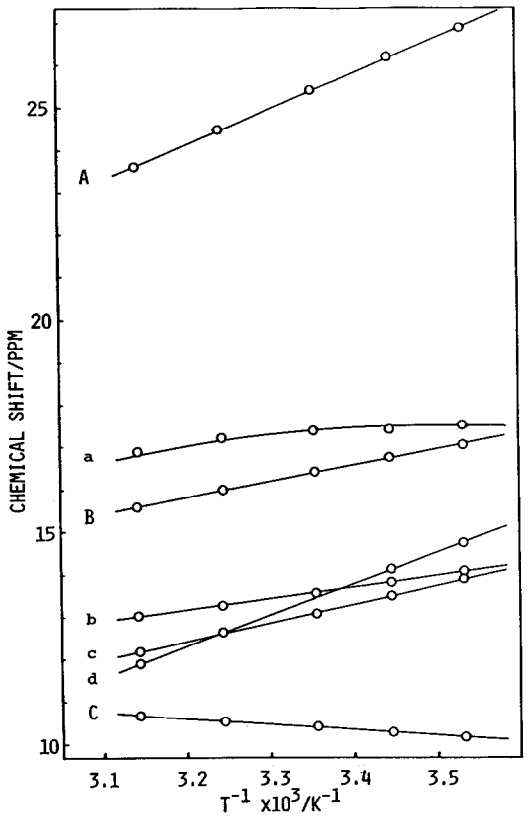

Fig.2. The Curie plots, the observed shifts vs reciprocal of the absolute temperature, for the downfield hyperfine shifted resonances of GJMbCN. The curvature of the plot for peak a is apparent, strongly supporting the assignment of this resonance to vinyl α-proton.

a characteristic cross-peak pattern in the COSY spectrum [23,24].

The NOESY spectrum of GJMbCN, p^2H 8.5, at 35°C is illustrated in A of fig.3 and region a is expanded in B. A direct inter-methyl dipolar coupling connectivity between heme methyl peaks B and C is observed and such connectivity is possible only between heme 1- and 8-CH_3 resonances as demonstrated in the met-cyano form of sperm whale skeletal muscle myoglobin (SWMbCN) [25]. The dipolar coupling connectivities, Y and Z, in B of fig.3 indicate that these vinyl β-proton resonances are oriented in the close proximity of the heme methyl group of peak B. Therefore peak B must arise from 1-CH_3 and hence peaks C and a are assignable to 8-CH_3 and α-proton of the 2-vinyl group, respectively. It has been revealed that the heme methyl proton resonance pattern crucially depends on the orientation of proximal histidyl imidazole plane relative to the heme [26–28]. The similarity in the heme methyl proton spectral pattern between GJMbCN and SWMbCN strongly argues that the orientation of the proximal histidyl imidazole plane relative to heme in GJMb is similar to that in SWMb, i.e., the imidazole ring is oriented roughly along the N_{II}-Fe-N_{IV} axis (N_{II} and N_{IV} are nitrogen nuclei of the pyrrole II and IV, respectively) [29]. Hence peak A is most likely to be 5-CH_3 and then the 3-CH_3 resonance appears to be resonating in the diamagnetic chemical shift region.

The analyses on the cross-peaks shown in B of fig.3 not only provide the specific assignment of vinyl β-proton resonances but also allow the determination of the vinyl group orientation with respect to the heme plane. The dipolar coupling connectivity of the 2-vinyl α-proton resonance with the β-proton resonance, indicated by X, but not with the other β-proton resonance indicates that the former resonance is assigned to *cis* β-proton and therefore the latter is *trans* β-proton. The connectivity between 1-CH_3 and *trans* β-proton, indicated by Y in B of fig.3, is stronger than that between 1-CH_3 and *cis* β-proton, Z, dictating that 1-CH_3 is closer to the *trans* β-proton than to the *cis* β-proton and the 2-vinyl group in GJMbCN does not undergo a flip-flop motion as detected in cytochrome b_5 [9] but is fixed in the so-called *cis* in-plane orientation as shown in C of fig.3. Therefore the 2-vinyl group orientation of GJMbCN was found to be the same as that of SWMb in the crystal [4–7]. The orientation of the 4-vinyl group is not determined at present because of the inability to locate the 3-CH_3 resonance whose dipolar coupling connectivities with 4-vinyl proton resonances are directly interpretable in terms of the 4-vinyl group orientation. ^1H-^{13}C COSY spectrum [30–32] would be useful to detect the 3-CH_3 resonance in GJMbCN.

Although the application of 2D NMR techniques to the paramagnetic systems is limited by the difficulties arising from the loss of the magnetization during the evolution and the mixing periods in the 2D NMR pulse sequences due to the rapid paramagnetic relaxation and the low digital resolution due to the large chemical shift range for an instrumentally limited data point, which obscures the fine structure of the cross-peak pat-

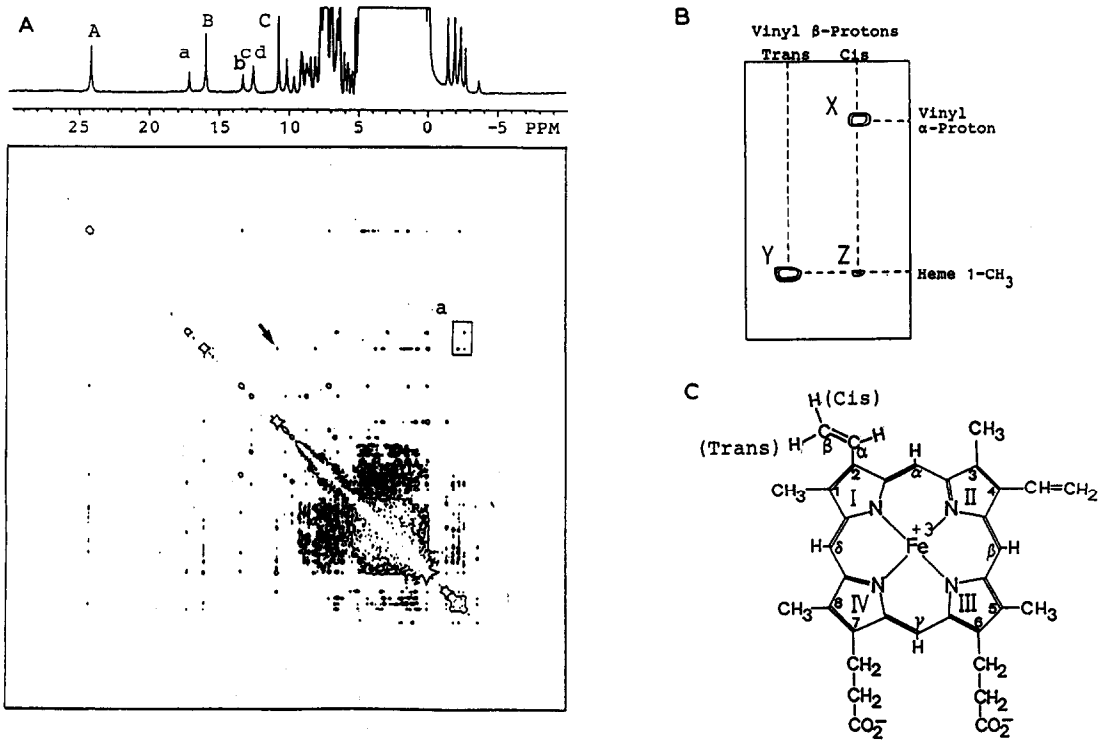

Fig.3. (A) NOESY spectrum of GJMbCN, p^{2}H 8.5, at 35°C. The inter-methyl dipolar coupling connectivity, indicated by an arrow, is detected between peaks B and C. (B) Expanded spectrum of region a in (A). (C) Structure of hemin and the heme 2-vinyl group is shown in the orientation determined from the cross peaks shown in (B).

tern, 2D NMR is still found to be useful for studying certain paramagnetic molecules in solution [9,33]. It has been shown above that the orientation of the functionally important heme peripheral vinyl group in Mb can be determined clearly by the combined use of COSY and NOESY and the procedures presented here should be applicable to other hemoproteins.

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