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Myoglobin 시안 단백질에 포함된 Heme에 대한 전자 및 분자구조 규명을 위한 ^{13}C NMR분석

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^{13}C NMR Analysis for the Characterization of Heme Electronic/Molecular Structure in Horse Myoglobin Cyanide

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요 약: 미오글로빈 시안착물(MbCN) 단백질에 대한 NMR의 HMQC 연구는 수소와 결합된 상자기성 heme 탄소 시그날의 완전한 지정을 가능토록 해준다. 이러한 상자기성 MbCN에 대한 HMQC 실험의 적용은 heme시그날뿐만 아니라 상자기성 아미노산에 대해 결합된 수소와 탄소간의 coherence를 지정하여주며 자연존재량 ^{13}C시그날의 지정이 모든 low-spin 상자기성 heme단백질에서도 가능하다. 이러한 시그날 지정 전략은 정자기성 영역에서 공명하는 수소 시그날의 지정을 위해 사용되는 NOE에만 의존하는것 보다 훨씬 명확한 시그날지정이 가능하다. 2,4-비닐기의 α-탄소들과 7-프로피온기의 β-탄소에서 특이한 anti-Curie형태를 보이는 것은 그들이 heme평면에 존재하고 있지 않다는 증거가 된다. Proximal His에 의해 유도된 heme의 전자 및 자성의 비대칭은 heme탄소 시그날공명이 25°C에서 250 ppm의 범위에 이르도록 한다. 이러한 heme 탄소 시그날 공명은 미오그로빈 heme의 전자구조를 분석하는데 있어서 수소 시그날의 공명보다 더욱 민감한 증거로 작용할수 있다.

ABSTRACT: The reverse detection heteronuclear multiple quantum coherence, HMQC study of met-cyano complex of horse myoglobin(MbCN) has provided the complete assignment of hyperfine shifted resonances of heme carbons attached with proton(s). The application of HMQC experiment to the paramagnetic low-spin MbCN gives clear ^{1}H and ^{13}C coherences for the paramagnetic amino acid residues as well as heme side chains, and can be extended to the low-spin paramagnetic hemoprotein derivative for the assignment of natural abundance ^{13}C resonances. This assignment strategy can avoid possible ambiguities that may result from the sole utilization of ^{1}H nuclear Overhauser effect for the assignment of heme ^{1}H signals resonating in the diamagnetic region. The resulting 2,4-vinyl α-carbons and 7-propionate β-carbon follow anomalous anti-Curie behavior, and are indicative of incoplanarity with heme plane. Magnetic/electronic asymmetry of heme induced by proximal histidine(His) makes spread that the hyperfine shifted heme carbon resonances over the range of 250 ppm at 25°C. These heme carbon resonances would be the much more sensitive probe than those of proton resonances in analyzing the nature of heme electronic structure of myoglobin.

Key words: ^{13}C NMR, HMQC, myoglobin, heme, hyperfine-shift

1. INTRODUCTION

Nuclear magnetic resonance (NMR) plays one of the major role for elucidating the solution structure of proteins[1] and significant structural details have been derived for myoglobin (Mb) complexes. While the sterochemical structural information content of paramagnetic Mb derivatives may be less than that in diamagnetic complexes, the hyperfine shifts provide unique additional information on the electronic/magnetic properties of the heme that are inaccessible in diamagnetic systems.[2,3] Considerable effort has been expended toward characterization of the ^{1}H NMR spectra for paramagnetic hemoproteins.[3] The information content of ^{1}H hyperfine shifts in low-spin ferric hemoprotein is extremely rich, since this state exhibits both contact and dipolar shift provides both molecular structural details for non-coordinated amino acid residues and information on the magnetic properties of the iron.[3]

The assignment of ^{1}H heme resonances for sperm whale metMbCN has been reported on the basis of isotope labeling,[4] the steady-state nuclear Overhauser effect.[5,6] The successful application of 2D techniques(NOESY and COSY) for sperm whale metMbCN has recently provided the complete assignment of ^{1}H heme signals and the majority of paramagnetic proteins($r_{Fe\text{-}H}$<7.5Å).[7] These unambiguous assignment led to the determination of new magnetic coordinate for the heme-protein interactions that are the major determinants of the electronic/magnetic properties of the heme.[8] To date, those data have been obtained and interpreted mainly from ^{1}H NMR spectra. The ^{13}C chemical shifts for heme substituents can, in principle, provide additional valuable information on heme electronic and molecular structure, particularily in paramagnetic Mb derivatives.[9~11] 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 provide some insight into the origin of ^{13}C chemical shifts due to the electronic asymmetry of heme iron.[12~15] Also, it assist the cross assignments of the less resolved signals by ^{1}H nuclear Overhauser effect due to the much larger ^{13}C chemical shift dispersion. Furthermore, determining the contact and pseudo-contact contribution to the hyperfine ^{13}C chemical shift would allow to estimate the unpaired electron spin density distribution in heme and axial ligand.[12-15]

In spite of a substantial senstivity advantage of HMQC over the conventional shift-correlation experiment, it, however, has not yet gained the popularity one might expect on the basis of intrinic advantages. Less attention has been directed toward HMQC experiment. This is due to the dynamic range problem that is introduced by the presence of large signals from protons that are not coupled to the low-γ nucleus (^{13}C) and the required suppression of these intense signals in a difference experiment. Also, this neglect is likely due to the belief that the severe line broadening that can accompany paramagnetism obscures the ^{1}H-^{13}C connectivities and renders the detection of coherence in an HMQC map experimentally impossible.

Natural abundance ^{13}C NMR studies have been reported for cytochrome c[16,17] and metMbCN.[9~11] They have been assigned using specfically ^{13}C enriched heme reconstituted protein, Distortionless Enhancement Polarization Transfer (DEPT) and conventional ^{1}H-^{13}C Heteronuclear Correlation Spectroscopy (HETCOR) which has much less sensitivity than HMQC. In earlier studies, hyperfine shifted carbon resonances arising from heme of metMbCN have not been fully and correctly identified.

We report on the complete assignments of hyperfine shifted heme carbons attached with proton(s) in horse metMbCN using one-shot HMQC experiment which detect the characteristic ^{1}H-^{13}C coherence of paramagnetic heme and those would be extremely valuable new probe for the characterization of the heme electronic/magnetic structure by ^{13}C NMR.

2. MATERIALS AND METHODS

Horse heart myoglobin was purchased from Sigma Chemical Co. as a lyophilized powder and used without further purification. Cyanometmyoglobin (metMbCN) was prepared by dissolution of the lyophilate in ^2H_2O containing 0.2 M NaCl and 20 mM

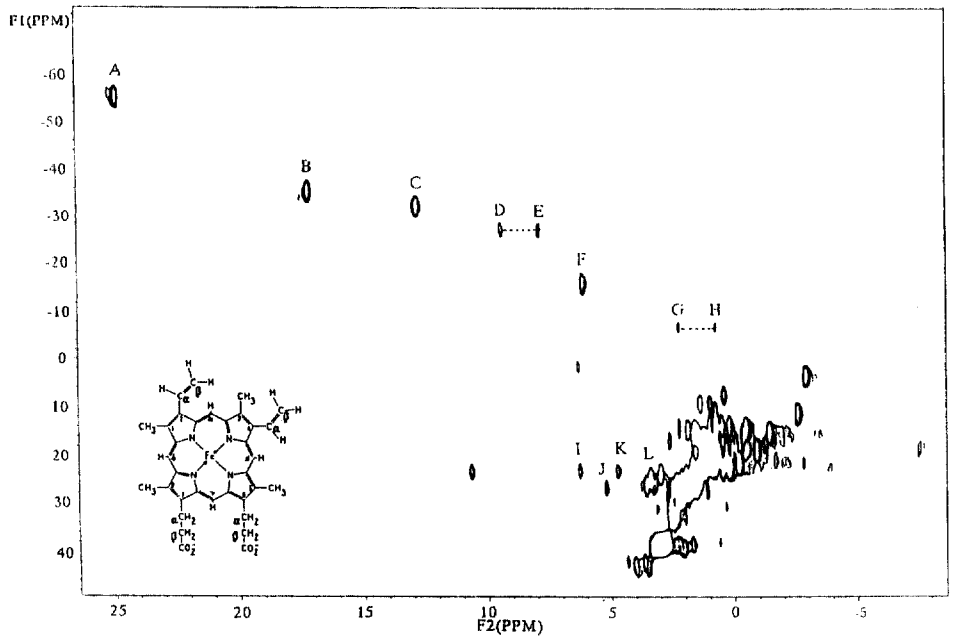

Fig. 1. A region of 600-MHz (^1H frequency) HMQC spectrum of horse metMbCN, pH 8.9, 45°C, showing ^1H-^{13}C coherence between −70~50 ppm (^{13}C dimension) window and −9~27 ppm (^1H dimension) region. The spectrum results from 4096×1024 data matrix and recorded ~10 hours. The structure and numbering system of the heme are given in the inset.

KCN. 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.[18] All protons not coupled to ^{13}C are inverted by the bilinear (BIRD) pulse in spite of the lower sensitivity by the negative nuclear Overhauser effect. The spectral width and number of points acquired were 40,000 Hz and 4096 complex points in ^1H (F2), 50,000 Hz and 128 real points in ^{13}C(F1). The initial data matrix was expanded to 4096×512 by linear prediction, and zero-filled to 4096×1024. Broad band decoupling with the Globally Optimized Alternating Phase Retangular Pulse (GARP) sequence was used during the acquisition period. 128 transients were accumulated per increment. The total acquisition time was ~10 hours. Solvent suppression was achieved by on resonance presaturation of the solvent signal. The spectra were acquired over the range 25~55°C with 10°C interval to determine the temperature dependence of all ^{13}C heme resonances. The phase-sensitive HMQC map shown in portions of *Figure* 1~2 was collected at 45°C by using States-Haberkorn method[19] for the nonacquired dimension. Spectra were processed with VNMR software (version 4.3) provided by Varian Associate. The data matrix was apodized with gaussian function in both dimensions. ^1H and ^{13}C chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)[20]

3. RESULTS AND DISCUSSION

The highly resolved upfield-shifted region (^{13}C dimension) of HMQC spectrum of horse metMbCN, pH 8.9, 45°C is shown in *Fig.* 1. This HMQC spectrum displays the scalar couplings of the ^1H-^{13}C hyperfine shifted resonances between the −70~50

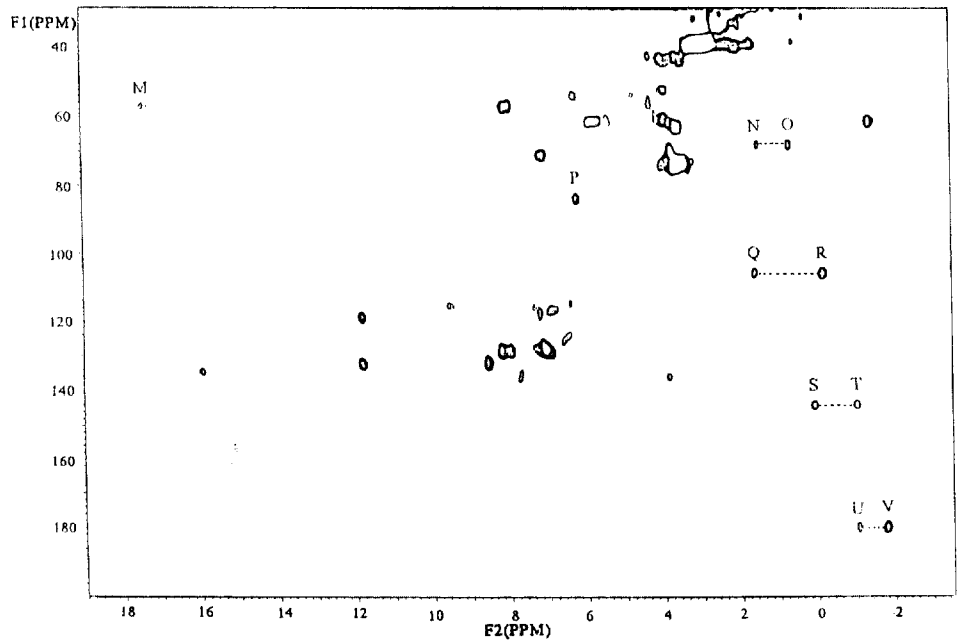

Fig. 2. A region of 600 MHz (^{1}H frequency) HMQC spectrum of horse metMbCN, pH 8.9, 45°C, showing ^{1}H-^{13}C coherence between 30~200 ppm (^{13}C dimension) window and −3~19 ppm (^{1}H dimension) region. The spectrum results from 4096×1024 data matrix and recorded ~10 hours.

ppm (^{13}C region) window and the −9~27 ppm (^{1}H region) range. This spectrum demonstrates the ability to map covalent connectivities between heme carbon and attached proton (s) with a much higher sensitivity advantage over the conventional ^{1}H-^{13}C HETCOR experiment within a short period of time (less than 10 hours). The hyperfine-shifted ^{1}H resonances of heme for horse MbCN should be at first assigned unambiguously to locate the ^{13}C heme signals. These ^{1}H heme signals of horse MbCN were assigned using NOESY and double quantum filtered (not shown) on the basis of earlier report documented on sperm whale MbCN,[7] and their ^{1}H chemical shifts for heme signals of horse MbCN are illustrated in *Table 1*. Although horse Mb has a little different peptide sequence with sperm whale Mb as a genetic variant, the sequence homology in heme pocket lead to the very similar hyperfine shifted ^{1}H chemical shift pattern between horse and sperm whale Mbs. Once the assignment of hyperfine shifted ^{1}H heme resonances for horse MbCN were confirmed, heteronuclear coherence spectra shown in *Fig.* 1 and 2 led to locate ^{13}C heme resonances from the known ^{1}H signals. Correlation peaks, A~C and F were assigned to 5-CH_3, 8-CH_3, 1-CH_3 and 3-CH_3, respectively, from the known ^{1}H chemical shifts of heme methyl groups. Previous assignments for heme methyl group of sperm whale MbCN using conventional ^{1}H-^{13}C HETCOR were consistent with our data set from HMQC except 3-CH_3 group.[10] This earlier wrong assignment for 3-CH_3 group may be due to intrinsic low sensitivity of conventional ^{1}H-^{13}C HETCOR and that seems to be a limitation to the application of classical HETCOR to paramagnetic hemoproteins. Correlation peaks, D and E corresponds to 6-propionate α-protons in ^{1}H (F2) dimension indicate the covalent connectivities between 6-propionate α-protons and α-carbon in ^{13}C(F1) dimension. Correlation peaks, G and H which corresponds to 7-propionate α-protons in F2 (^{1}H) dimension show a 7-propionate α-carbon. Correlation peaks I, J, K and L which correspond to heme meso protons in F2 dimensions indicates γ-, δ-, α- and β-mesos ^{13}C chemical shifts in F1 dimension, respectively. Although it is hard to locate heme meso protons resonating in di-

Table 1. Assignment of ^{1}H and ^{13}C chemical shifts of heme resonances in horse metMbCN at 25°C and pH 8.9

Label[a]	Chemical shift (^{1}H)[b]	Chemical shift (^{13}C)[c]	Assignment
A	27.05	−59.9	5-CH_3
B	18.47	−38.5	1-CH_3
C	13.40	−34.0	8-CH_3
D	9.36	−28.9	6-P_α
E	7.63	−28.9	6-P_α
F	5.28	−14.7	3-CH_3
G	1.45	−5.8	7-P_α
H	0.31	−5.8	7-P_α'
I	6.58	25.2	γ-meso
J	5.90	29.2	δ-meso
K	5.22	23.4	α-meso
L	3.89	25.8	β-meso
M	17.83	53.8	2-V_α
N	1.47	69.0	7-V_β
O	0.63	69.0	7-V_β'
P	5.73	81.1	4-V_α
Q	1.58	110.4	6-P_β
R	−0.51	110.4	6-P_β'
S	−0.59	147.6	4-$V_{\beta c}$
T	−1.77	147.6	4-$V_{\beta t}$
U	−1.51	186.1	4-$V_{\beta c}$
V	−2.42	186.1	4-$V_{\beta t}$

[a]As labeled in Figure 1 and 2, [b]In ppm at 25°C, pH 8.9 from DSS, [c]In ppm at 25°C, pH 8.9 from DSS

amagnetic region, NOESY cross peaks between meso protons and neighboring heme substituents at diverse temperatures (not shown) provides unambiguous assignment of heme meso protons for horse MbCN. The highly resolved downfield shifted region (^{13}C dimension) of HMQC spectrum of horse metMbCN, pH 8.9, 45°C is shown in *Fig.* 2. This HMQC spectrum displays the scalar couplings of ^{1}H-^{13}C hyperfine shifted resonances between the 30~200 ppm (^{13}C region) window, the −3~200 ppm (^{13}C region) window and the −3~19 ppm (^{1}H) range. Correlation peaks, M and P which correspond to 2- and 4-vinyl α-protons in F2 dimension give a 2- and 4-vinyl α-carbons chemical shifts. Correlation peaks, S and T which correspond to 4-vinyl β-protons at 45°C gives a 4-vinyl β-carbon chemical shift. Correlation peaks, U and V which correspond to 2-vinyl β-protons at 45°C give a 2-vinyl β-carbon chemical shift. Sankar, *et al.*[9] have previously documented on the assignments of the vinyl carbon resonances in the spectra of various complexes of Mb obtained by using Mb reconstituted with specifically ^{13}C labelled heme at α- or β- carbons of heme vinyl groups. However, as shown in *Fig.* 2, HMQC spectrum clearly shows ^{1}H and ^{13}C scalar connectivities between heme vinyl carbons and attached proton(s) without relying on the introduction of ^{13}C enriched hemins through tedious synthetic routes.[21] Correlation peaks, N and O which correspond to 7-propionate β-protons in F2 dimension give a 7-propionate β-carbon chemical shift. Cross peaks, Q and R which correspond to 6-propionate β-protons at 45°C give a 6-propionate β-carbon chemical shift.

As described above all heme carbon resonances attached with proton (s) 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 shift except meso carbons. Anomalous chemical shift pattern of four meso carbons would be due to the dominant dipolar contribution.[8]

The Curie plots, the observed heme ^{13}C chemical shifts vs. reciprocal of the absolute temperature is displayed in *Fig.* 3. The slope of the ^{13}C chemical shift change for the variable temperature dictates Curie behavior with straight lines except 2,4-vinyl α-carbons and 7-propionate β-carbon. These anti-Curie behavior for 2,4-vinyl α-carbons and 7-propionate β-carbon can be rationalized by incoplanarity between heme plane and side chains, or thermal expansion for specific site of heme cavity.[9,17]

A detailed attempt to separate relative contributions to the paramagnetic carbon shifts should await the unequivocal assignments of the heme pocket amino acid residues. ^{13}C resonances assignment of paramagnetic amino acid residues located in heme pocket are currently being explored. 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., metmyoglobin derivatives, diverse cytochromes) and can be quite effectively utilized to assign those reso-

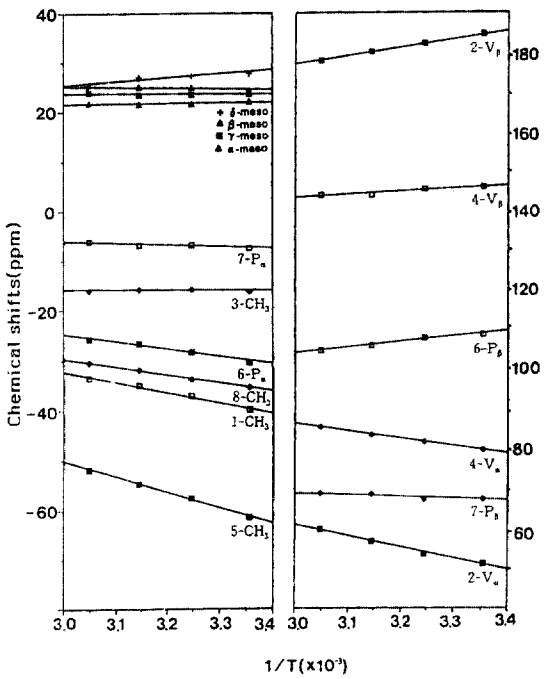

Fig. 3. The Curie plots, the observed chemical shifts vs. reciprocal of the absolute temperature, for the hyperfine shifted ^{13}C heme resonances of horse metMbCN.

nances from the known ^{1}H assignment. Such studies are currently in progress.

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