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Heme proton resonances assignments based on nuclear Overhauser effect

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Abstract : NMR signals of two hemes were assigned to particular hemes in the crystal structures by nuclear Overhauser effect experiments. The results showed that the hemes with the highest and lowest redox potentials in the one-electron reduction process correspond to the hemes I and IV in the crystal structure.
Keywords : Heme proton, NOE

INTRODUCTION

Cytochromes have been the subject of a large number of NMR studies not only because of their important biological roles as electron transfer proteins, but also due to the attractive features in NMR spectra. In the oxidized from, the paramagnetism of the iron causes heme and protein proton resonances to be spread over a wide range by Fermi contact and/or dipolar interaction. The natures of heme protons and ligands of several cytochromes have been determined by analysis of these well-resolved resonances[1]. The most useful information to be extracted from these resonances is the nature of the interaction between cytochromes and other electron transfer proteins. In the case of cytochrome c_3, a tetraheme protein isolated from sulfate-reducing bacteria *Desulfovibrio vulgaris Miyazaki F (Dv Miyazaki F)*, the intramolecular and intermolecuiar electron transfer rates have been found

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to be faster or slower with respect to NMR time scale, respectively, and a strong cooperative interaction has been shown for a pair of hemes[2-4]. In order to elucidate the cooperative interaction in terms of conformational parameters, heme resonances have been partially assigned by a nuclear Overhauser effect (NOE) experiment.

METHODS

JEOL-400s and JEOL-500s NMR spectrometers were used. The nuclear Overhauser effect experiments were performed with typical 0.2 sec preirradiation and the accumulation of 8,000 transients. The difference spectrum was obtained by subtracting the on-resonance FID the off-resonance FID.

RESULTS AND DISCUSSIONS

Table 1 shows the carbon-carbon distances among heme methyl groups of cytochrome c_3 of *Dv . Miyazaki F* in the crystal structure[5]. Designation of heme methyl groups in heme *c* was shown in Fig. 1 and ring methyl positions are shown in Fig. 2. Since the C-H bond generally has a length of about 1.1 Å, the longest interproton distance that can give NOE is about 5 Å. NOE among intraheme methyl group protons is expected only between position 1 and 8.

Table 1. C-C distances (below 10 Å) among the intraheme heme methyl groups obtained from X-ray crystallographic studies

Ring methyl groups	Distance (A) 1-8	1-3	3-5	5-8
Heme I	5.313	8.064	8.036	> 10
Heme II	5.328	7.904	7.971	9.960
Heme III	5.253	7.915	8.054	9.880
Heme IV	5.361	8.056	7.984	9.939

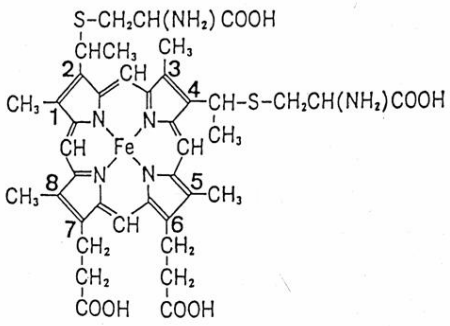

Fig. 1. Designation of heme methyl groups in heme *c*.

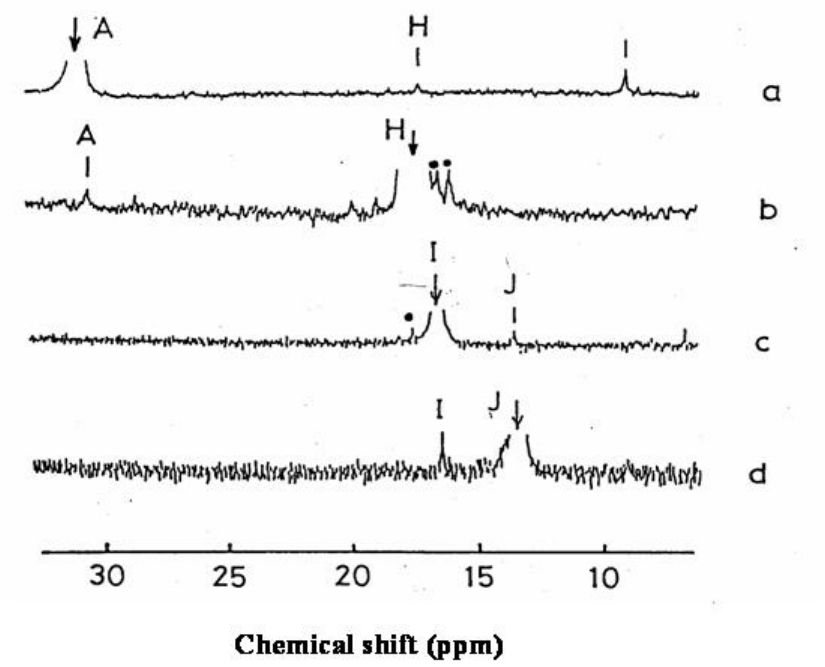

Fig. 2. NOE difference spectra of cytochrome c_3 in the fully oxidized state at 19 °C (a) and 30 °C (b,c,d). The arrows indicate the irradiated positions. A closed circle denotes power spillage. In the case of (a), the measurement was carried out at a lower temperature because a single proton signal at around 9.6 ppm, which gives an NOE signal, overlapped with heme with heme methyl signal M at 30 °C.

NOE difference spectra with of methyl proton of signal A, H, I and J are presented in Fig. 2. On irradiation at the methyl proton at signal A, weak NOE signals were observed at the positions of heme methyl signal H (Fig. 2a). The NOE signal at about 9.6 ppm originates from a single proton signal. The irradiation of proton corresponding to signal H also gives an NOE signal at position of signal A (Fig 2b). Since the intraheme NOE can be observed only between the methyl groups at 1 and 8 positions[6], we can assign signals A and H to either 1 or 8 methyl groups of heme 1. On irradiation of proton at signal I, a strong NOE was observed at signal J and vice versa (Fig. 2c and Fig. 2d). Since there is no overlapping at signals I and J, and they belong to heme 1 and 3, respectively, we can conclude that this is the interheme NOE. The crystal structure of this protein[5] shows that the shortest interheme methyl carbon distance is 0.417 nm and the second shortest one is 0.534 nm. The former is the only pair that can give rise to a strong NOE signal. Since the experiments mentioned above showed that signal I is neither methyl 1 nor 8, signal I and J can be assigned to the methyl 5 of heme I and methyl 1 of heme IV in the crystal structure, respectively. It leads to the assignment of hemes 1 and 3 to those I and IV in the crystal structure, respectively. This assignment is consistent with that inferred on the basis of the crystal structure of heme groups and the results of electrochemistry[7] but contradicts that by EPR[8], which attributed heme 3 to heme II. The assignment of heme 1 is in accord with that for *D. desulfuricans*, Norway, cytochrome c_3 on the basis of chemical modification and electrochemistry[9].

NOE results showed that the hemes with the highest and lowest potentials in the first reduction step (heme 1 and heme3) are the hemes I and IV in the crystal structure, respectively. Heme I has the least exposure to the solvent and is surrounded by the highest positive charge density among four hemes[5].

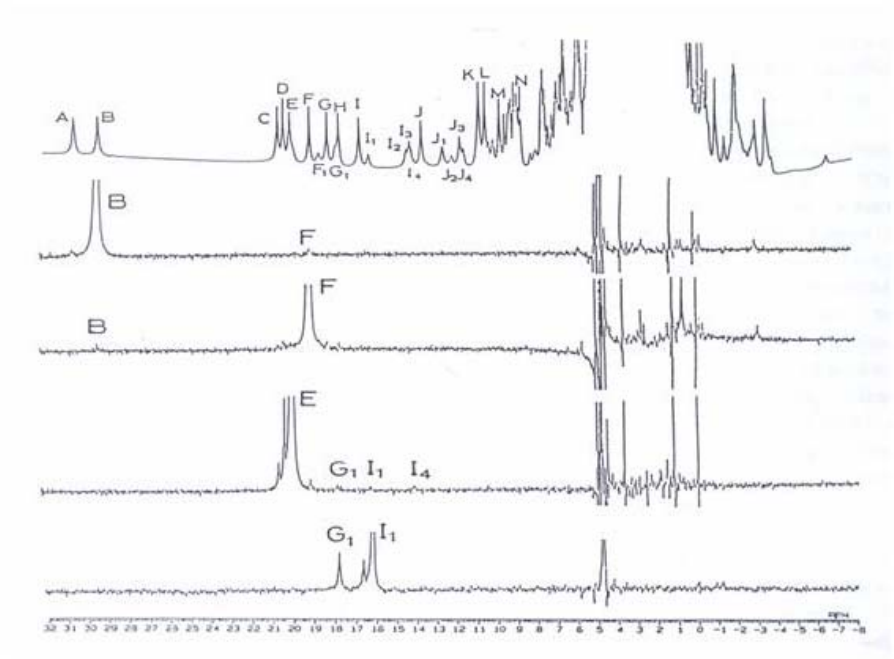

Fig. 3. NOE different spectra of resonances B, F, E and I_1.

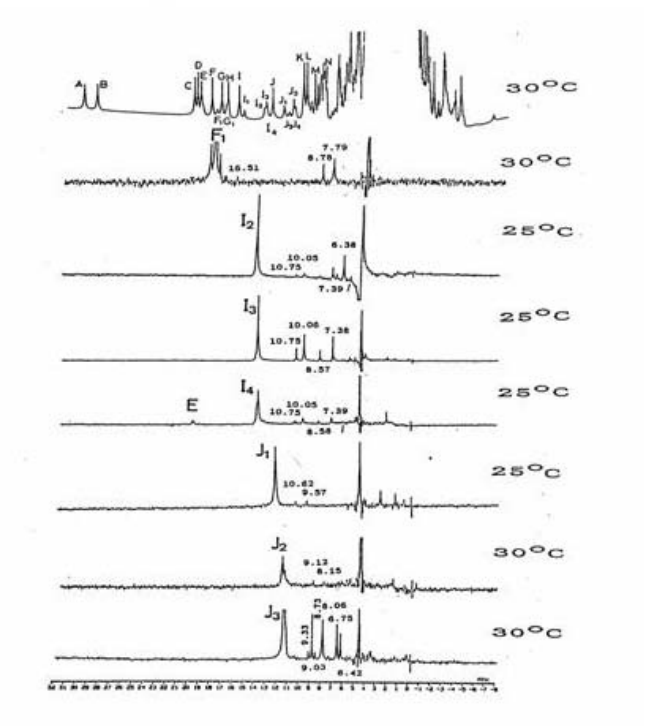

Fig 4. NOE difference spectra observed from one proton signals.

Fig 3 and Fig 4 give the NOE difference spectra on irradiation at signals B, F, E and I_1. NOEs between signal B and F were observed after irradiation at signal B and vice versa. Since resonances B and F have been classified to heme 2, this NOE can be ascribed to an intraheme one. Thus, B and F can be assigned to the methyl groups either in position 1 or 8.

Irradiation at signal E gave rise to NOEs with similar intensity at 17.62, 16.07 and 13.94 ppm, which are named to G_1, I_1 and I_3+I_4, respectively. The reverse effect was confirmed by irradiation of I_1. Signals G_1 and I_1 have one proton intensity. The signals I_3+I_4 have two-proton-intensity and were separated to two peaks by shifting the temperature from 30 °C to 25 °C. NOE was confirmed at signal I_4. A strong nuclear Overhauser effect of signal G_1 was observed on irradiation of signal I_1. The percentages here for difference spectra are determined by taking the ratio of the integrated intensity of the peak displaying the NOE divided by the number of protons comprising it to the integrated intensity of the irradiated peak divided by the number of protons comprising it[10]. Since this effect is extrem-

ely strong and signal G_1, I_1 appear in the low field region, they can be assigned to germinal protons of a propionic acid. Their chemical shifts are in the reasonable range for an oxidized low-spin cytochrome. Therefore, ring methyl E can be assigned either in position 5 or 8. Since no NOE between intraheme methyl groups was observed on irradiation at signal E, it might possibly be ring methyl 5. thus I_1 and G_1, become two α-protons of 6-propionic acid of heme 4. Results are summarized in Table 2.

Table 2. NOE assignments of partial heme resonances

Peak designation	Chemical Shift (ppm)	assignment	Heme (NMR)	Heme (crystal)
A	30.51	1 or 8 CH_3		
H	17.53	1 or 8 CH_3	1	I
I	16.45	5 CH_3		
B	29.31	1 or 8 CH_3	2	-
F	18.98	1 or 8 CH_3		
J	13.52	1 CH_3	3	IV
E	19.90	5 CH_3	4	-
G_1	17.62	6 H α	4	-
I_1	16.06	6 H α		

It is known that the orientations of the four hemes in the crystal structures are identical almost to cytochrome c_3 from *Dv. Miyazaki F*, and *Dd. Norway*, in spite of their low sequential homology. Two of four hemes are located very close to each other at an almost right angle with an intervening phenylalanine, which is conserved in all cytochrome c_3 so far examined. A specific interaction between these hemes was anticipated by crystallographers[5,11]. NOE results showed that heme 3 in the NMR spectrum is heme IV in the crystal structure, and interacting potential I_{23} has a specific nature. Although the assignment of heme 2 is not yet established, it is highly probable that the positive interheme interaction has something to do with the specific conformation of hemes III and IV in the crystal structure.

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