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Partial Assignment of Heme Groups of Cytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F by ^{1}H-NMR

Jang-Su Park* and Shin Won Kang

Departmment of Chemistry, College of Natural Sciences, Pusan National University, Pusan 609-735

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The ^{1}H-NMR signals of the heme methyl, propionate and related chemical groups of cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F (*D.v.* MF) were site-specifically assigned by means of 1D-NOE, 2D-DQFCOSY and 2D-TOCSY spectra. They were consistent with the site-specific assignments of the hemes with the highest and second-lowest redox potentials reported by Fan *et al.* (*Biochemistry*, **29**, 2257-2263 1990). The site-specific heme assignments were also supported by NOE between the methyl groups of these hemes and the side chain of Val-18.

Introduction

Cytochrome c_3 is a unique class of heme proteins which contain four hemes in a single polypeptide and show very low redox potentials[1]. Crystal structures of cytochrome c_3 from *Desulfovibrio desulfuricans* Norway and *D. vulgaris* Miyazaki F have been reported[2,3]. Cytochrome c_3 is of great interest not only from a biological point of view but also because of its peculiar physicochemical properties. Redox potentials are one of the important parameters for the electron transfer.

*To whom correspondences should be addressed.

Macroscopic and microscopic redox potentials were determined for a series of cytochrome c_3[4-6]. For elucidation of the redox potentials, it is essential to assign them to each hemes in the crystal structure. Gayda *et al.* assigned the third highest redox potential of cytochrome c_3 of *D. vulgaris* Miyazaki F using electron spin resonance (ESR) study[7]. Fan *et al.* assigned the highest and third highest redox potential of the same protein by the use of nuclear magnetic resonance (NMR)[8], which contradicted the assignment by ESR. The assignment was in conflict between chemical modification[9] and ESR[10] for *D.d.N* cytochrome c_3. A complete assignment of *D.v.* MF cytochrome c_3 was performed by NMR in this work. The result supported the partial assignment by Fan *et al.*

Expermeantal

Desulfovibrio vulgaris Miyazaki F was cultured in the medium C[1]. Cytochrome c_3 was purified according to the reported procedure[11]. The purity index (A_{552} (red)/A_{280} (ox)) of the final sample was over 3.0. The purity was also confirmed by SDS-polyacylamide gel electrophoresis. 400 MHZ ^{1}H-NMR spectra were obtained at 30℃ with a Bruker AM-400 NMR spectrometer in a 30 mM phosphate buffer solution (p^2H 7.0). Chemical shift are presented in parts per million relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). In the nuclear Overhause effect (NOE) experiments, 16transients were accumulated on- and off-resonance irradiation for 0.5s, alternately. In total 4800 transients were accumulated for each case, unless started otherwise. An NOE difference spectrum was obtained from these free-induction decays. Two-dimensional DQF-COSY spectra were measure with the data size of 512×2048, and spectral width of 25000 Hz. Two-dimensional TOCSY (HOHAHA) spectra were measured with the data size of 512×2048, spectral width of 8064 Hz and mixing time of 26.6 ms. Data processing was done with sine bell sequared window.

Results and Discussion

A 400 MHz ^{1}H-NMR spectrum of ferricytochrome c_3 is shown in Figure 1. As reported earlier[8], thirteen heme methyl signals can be observed separately in the downfield region. They are labeled alphabetically from the downfield. They were classified to four hemes on the basis of the electron distribution probabilities in the five macroscopic oxidation states[8], namely,

heme 1; A, H, I and K,
heme 2; B, F, G and M,
heme 3; C, D and J,
heme 4; E and L,

where hemes were numbered according to the order of the major reduction. La Mar and his coworker reported that the nuclear Overhouse effect (NOE) between the methyl protons at 1 and 8 positions of a paramagnetic heme can be used for the assignment of methyl signals[12]. The chemical structure of a *c*-type heme is given in Figure 1. NOE experiments were carried out by irradiation the heme methyl signals A through H one by one. As shown in Figure 2, intraheme NOEs were observed for the pairs of A and H, and B and

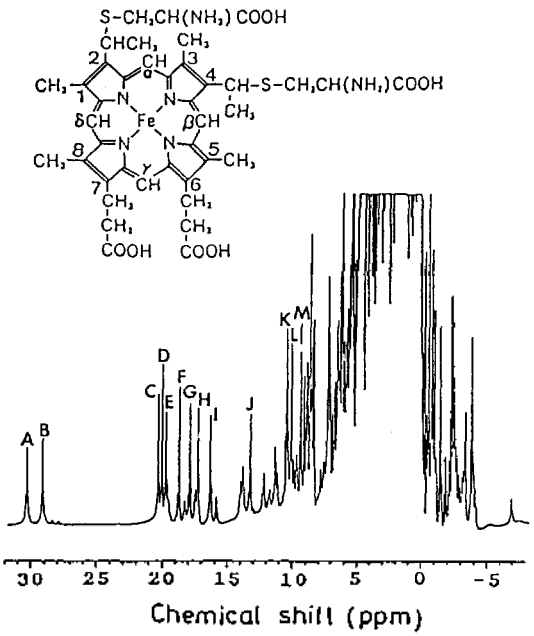

Figure 1. A 400 MHz ^{1}H-NMR spectrum of ferricytochrome c_3 from *D. vulgaris* Miyazaki F at 30℃. The heme methyl signals were labeled alphabetically. The chemical structure of a *c*-type heme and the labels of the porphyrin carbons are given on top.

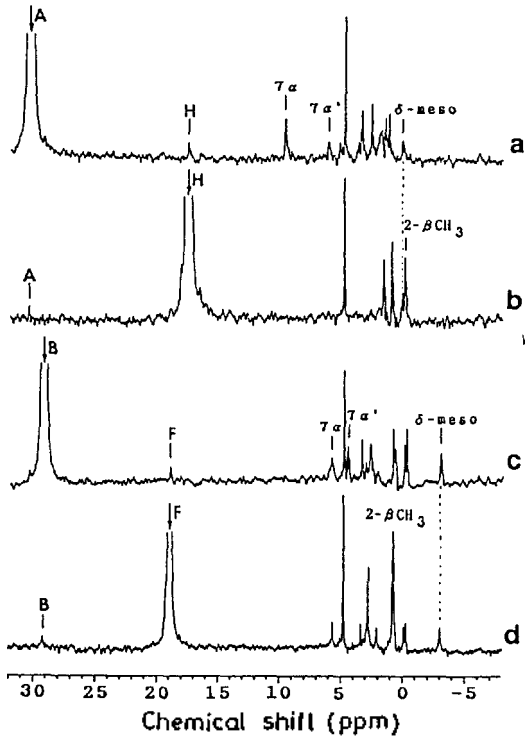

Figure 2. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. Definition of the labels is given in Figure 1. 7α denotes the α-methylene proton of the propionate at 7 position.

F. The former was already reported by Fan *et al*[8]. Thus, it can be concluded that signals A, B, F and H can be assigned to heme methyl groups at either 1 or 8 positions. Furthermore, NOE was observed at the α-methylene signals of pro-

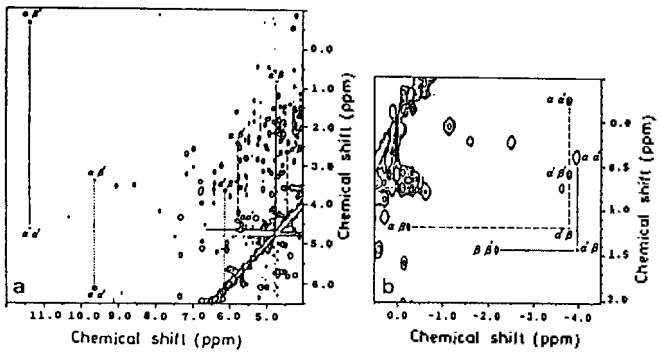

Figure 3. Sections of the two-dimensional TOCSY (HOHAHA) spectrum of ferricytochrome c_3 at 30℃. a; the dotted and broken lines connect αCH-βCH cross-peaks of 7-propionate of hemes 1 and 2, respectively. Solid lines connect those of 6-propionate of heme 3. b; the broken and solid lines connect αCH-βCH cross-peaks of 6-propionate of hemes 1 and 2, respectively.

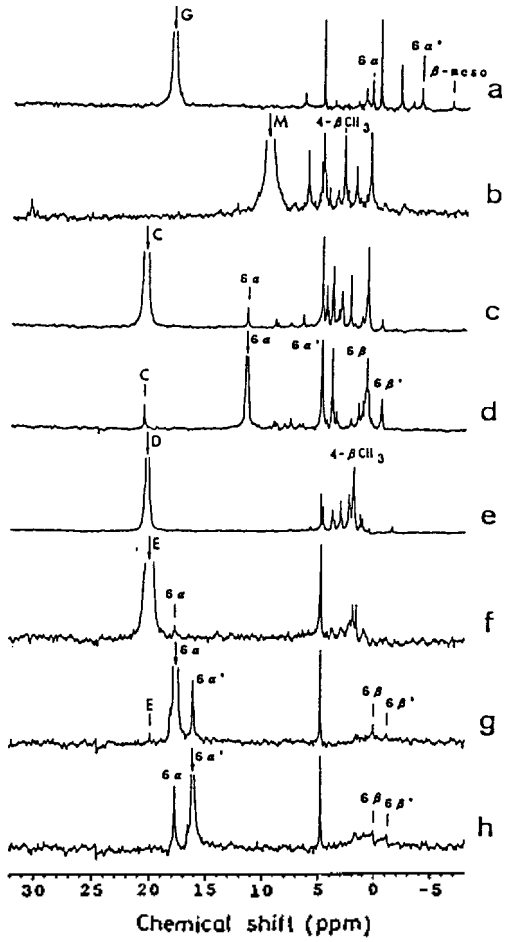

Figure 4. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. Definition of the labels is given in Figure 1. 6α and 6β denote the α-and β-methylene protons of the propionate at 6 position, respectively.

pionate for signals A and B as can be seen in Figure 2. Therefore, signals A and B, and those F and H can be ascribed to methyl groups at 8 and 1 positions, respectively. The assignment of the propionate protons was confirmed by 2D-

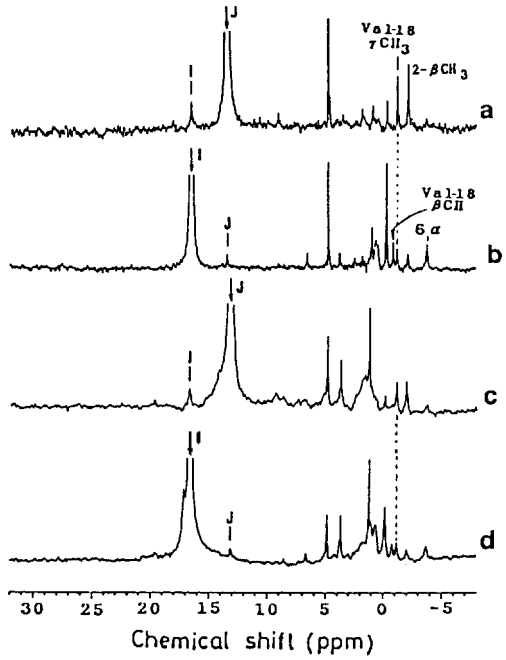

Figure 5. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. Definition of the labels is given in Figure 1. (a), (b); in aqueous solution, (c), (d); in the presence of 50% (v/v) of deuterated ethanol.

DQFCOSY and TOCSY spectra. A relevant portion of the 2D-TOCSY spectrum is presented in Figure 3. The common NOE signals between Figure 2a and b, and Figure 2c and d were assigned to δ-meso protons of hemes 1 and 2, respectively. The irradiation of signals I, G, C and E also gave rise to NOE signals at the α-methylene protons of propionates as can be seen in Figure 4. Their assignments were carried out on the 2D-DQFCOSY and TOCSY spectra. The connectivity is given in Figure 3. The assignment of the propionate signals observed on irradiation at signal E could also be confirmed by 1D-NOE experiments as shown in Figure 4g and h. Thus, signals I and G can be assigned to the methyl protons at 5 position (5-CH_3), which led to the assignment of K and M to 3-CH_3. Signals H, F, M, D and J gave NOE signals at β-methyl protons of the thioether bridges as shown in Figure 2, 4 and 5. The assignment of these signals was confirmed on the 2D-DQFCOSY and TOCSY spectra through the connectivity with α-methine protons.

Gayda *et al.* assigned heme 3 to heme II in the crystal structure on the basis of an ESR study of *D.v.* MF cytochrome c_3[7]. In contrast, Fan *et al.* assigned heme 3 to heme IV in the crystal structure by NMR[8]. To establish the assignment, this problem was examined further. The assignment by Fan *et al.* was proved by the interheme NOE between signals I and J. Since the shortest interheme methyl carbon distance in the crystal structure is 4.2 Å, between 5-CH_3 of heme I and 1-CH_3 of heme IV, and signal I was ascribed to either 3- or 5-CH_3, signal I and J were assigned to 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively[8], The NOEs observed for signal I and propionate, and J and β-methyl group of the thioether bridge are consistent with this assignment as far as heme methyl sites are concerned. However, the conformation in solution may be different from that in

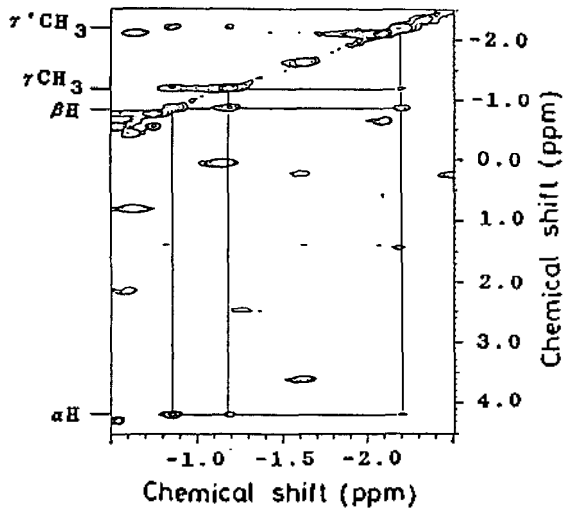

Figure 6. Sections of the two-dimensional TOCSY (HOHAHA) spectrum of ferricytochrome c_3 at 30℃, showing the J-connectivities for the side-chain spin system of a valine residue, which was assigned to Val-18 (see text).

crystal, which can change the interheme distance. Since, the crystal was obtained by adding ethanol to the solution[3], the effect of ethanol on the interheme NOE between signals I and J was examined. As can be seen in Figures 5c and d, the NOE did not disappear even in the presence of 50% (v/v) ethanol. Most cytochrome c_3 precipitated at 60-65% ethanol does not change the distance between the methyl groups represented by signals I and J significantly. Therefore, it is unlikely that the conformation in crystal is significantly different from that in solution as far as the interme-thyl distance of interest is concerned. Since irradiation of sign J did not induced NOE at C and D, they should be attributed to 5- and 3-CH_3, respectively. The signal at −6.68 ppm in Figures 1-4a was assigned to β-mesoproton because its irradiation gave NOE at signal G and 4-αCH. On irradiation at 4-αCH, NOE signals were observed at β-mesoproton and 4-βCH_3, which also provide the confirmation of the assignment of 4-βCH_3 signal. These assignments were summarized in Table 1. Furthermore, the NOE signal commonly observed in Figures 5a, b, c and d was assigned to the γ-methyl protons of a valine residue on the basis of the connectivities in 2D-DQFCOSY and TOCSY spectra. The latter is presented in Figure 6. In the crystal structure, the carbon of γ-CH_3 of Val-18 is located in the distances of 4.3 and 3.7 Å from 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively. No other shorter distance was found for between carbons of γ-CH_3 of valine and heme I and heme methyl groups. Accordingly, the common NOE signal at −1.21 ppm in Figure 5 can be assigned to γ-CH_3 of Val-18. This result also supports the site-specific assignment of heme groups by Fan *et al.* From the connectivity in 2D-NMR spectra, the NOE signal at −0.85 ppm observed in Figure 5b was assigned to the β-methine proton of Val-18 in the crystal structure is 4.0 Å, the appearance of NOE on irradiation at signal I also supports the assignment of Val-18 signals. In conclusion, all pieces of evidence we obtained are consistent with the assignment by Fan *et al.* but incompatible with that by Gayda *et al.* Signals I and J can be definitely assigned to the 5-methyl protons of heme I and 1-methyl protons of heme IV, respectively. The heme assignment is given in Table 1.

In order to characterize the other hemes, spin diffusion from 1-CH_3 of heme IV to other heme methyl groups was examined. The difference spectrum with the irradiation at signal J for 5 s is presented in Figure 7. The spin diffusion from signal J to those G, H, K, and L was observed. The assignment of ^{1}H-NMR signals carried out in this work fully supported the site-specific heme assignment by Fan *et al.*

Table 1. Resonance Assignments for Heme Protons in Ferricytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F at p^{2}H 7.0 and 30℃

Heme number in NMR	Signals	Assignment	Chemical Shift/ppm		Heme number in crystal
	A	8-CH_3	30.46		
	H	1-CH_3	17.47		
	I	5-CH_3	16.51		
1	K	3-CH_3	10.64		I
		δ-meso	0.09		
		6-αCH_2	0.23	−3.76	
		6-βCH_2	0.20	0.60	
		7-αCH_2	9.62	6.12	
		7-βCH_2	3.62	3.35	
		2-αCH	1.61		
		2-βCH_3	−0.18		
	B	8-CH_3	29.27		
	F	1-CH_3	18.92		
	G	5-CH_3	18.07		
	M	3-CH_3	9.60		
2		β-meso	−6.68		
		δ-meso	−3.05		
		6-αCH_2	0.41	−3.92	
		6-βCH_2	1.42	−2.20	
		7-αCH_2	5.79	4.46	
		7-βCH_2	2.65	2.41	
		2-αCH	−0.45		
		2-βCH_3	0.68		
		4-αCH	−2.99		
		4-βCH_3	2.87		
	C	5-CH_3	20.49		
	D	3-CH_3	20.21		
	J	1-CH_3	13.46		
3		6-αCH_2	11.36	4.67	IV
		6-βCH_2	0.67	−0.63	
		2-αCH	−0.65		
		2-βCH_3	−2.11		
		4-αCH	0.90		
		4-βCH_3	1.79		
	E	5 or 8-CH_3	19.91		
4	L	1 or 3-CH_3	10.30		
		6-CH_2	17.67	10.05	
		6-CH_2	0.08	−1.18	

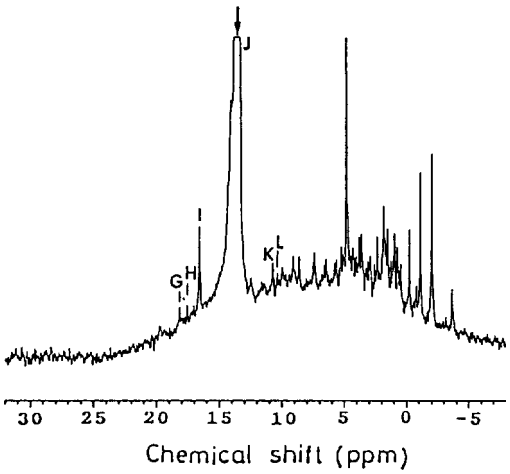

Figure 7. A spin-diffusion difference spectrum of ferricytochrome c_3. Signal J was irradiated for 5s. Otherwise, the conditions were same to the NOE difference spectra.

Since heme methyl groups represented by I, J, and K are located inside of the protein according to the assignment in Table 1, the spin diffusion from J to K is reasonable. However, heme methyl H is exposed to the solvent in the crystal structure. We checked the possibility of spill-over of the power by changing the power level of the irradition. But it was not the case. As can be seen in Figure 2b, heme methyl H is close to the β-methyl protons in the thioether bridge. This can provide a pathway of spin diffusion from the interior of the protein, because the structure of the bridge would be rigid.

Heme methyl G should be assigned to 5-CH_3 of either heme II or III in the crystal structure. While 5-CH_3 of heme II is exposed to the solvent, that of heme III is located in the interior of the protein. Moreover, 5-CH_3 of heme II has no rigid protons in the neighborhood in the crystal structure. Therefore, it is unlikely that there is a path of spin diffusion from J to G. It leads to a tentative assignment of signal G to 5-CH_3 of heme III. Therefore, we can tentatively assign hemes 2 and 4 in NMR spectra to those of III and II in crystal structure, respectively.

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Ring Transformations of Ethyl 4-Carbethoxy-5,6-dihydro-1-1-dioxo-2*H*-1,2,6-thiadiazin-5-ylethanoate into N-Alkyl-5-carbethoxy-2-pyridones

Chai-Ho Lee, Young Soo Chung, and Bong Young Chung*

Department of Chemistry, Won Kwang University, Chunbuk 570-749
**Department of Chemistry, Korea University, Seoul 136-701.* *Received May 6, 1993*

Treatment of ethyl 4-carbethoxy-5,6-dihydro-1,1-dioxo-2*H*-1,2,6-thiadiazin-5-ylethanoate (**3**) with alkylamines produced N-alkyl-5-carbethoxy-2-pyridones (**4**) in moderate yields.

Introduction

In recent years, we have been interested in the synthesis of some heterocycles containing sulfamide moiety[1] because they have been found to possess a wide range of biological activities such as anticonvulsant, hypoglycemic, antihypertensive, histamine-H_2-receptor antagonistic and herbicidal activities.[2] Unfortunately, little is known about the ring transfor-