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Specific Labeling of Cytochrome c_3 from *Desulfovibrio vulgars* Miyazaki F and its Assignment

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Abstract: In order to assign NMR signals, conditions for the specific labeling of cytochrome c_3 of *D. vulgaris* Miyazaki F through the culture in a minimal medium were established. Phenylalanine residue was specifically deuterated at more than 85% efficiency. Cytochrome c_3 has two phenylalanine residues. The signals of one phenylalane were missing and this was tentatively assigned to Phe20.
Key words: cytochrome c_3, NMR, minimal medium, specific labeling.

Cytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F is a tetraheme protein with a molecular weight of 14, 000. It is involved in the electron transport system of sulfate-reducing bacteria as a partner of hydrogenase. Although extensive investigations of this protein have been carried out by the use of a wide variety of physicochemical methods, the significance of four hemes has not yet been elucidated. Its four macroscopic and 32 microscopic redox potentials were estimated on the basis of electrochemical and NMR results (Niki *et al.*, 1987; Fan *et al.*, 1990a; Fan *et al.*, 1990b; Park *et al.*, 1995). The crystal structure of this protein was reported at 1.8 Å resolution by Higuchi *et al.* (1984). The crystal data enable us to investigate the relationship between the structure and redox behavior of this protein.

In the crystal structure, the shortest and longest inter iron distances are 11.3 and 18.1 Å, respectively. All of the fifth and sixth ligands of the four hemes are imidazole rings of histidine residues. The cytochrome c_3 molecule carries two phenylalanine, three tyrosine and one nonligated histidine residues, which are located in the vicinity of heme cavities, Tyr65 (tyrosine at the 65th position of the amino acid sequence) and Tyr66 are found in the vicinity of heme I, and Phe76 and His67 are close to heme II. Intervened between hemes III and IV is Phe20, which is conserved in all the sequences of cytochrome c_3 examined so far. Tyr43 is located in the vicinity of heme IV. Some of these aromatic rings may be involved in inter- and intramolecular electron transfer.

If proton NMR signals of these aromatic rings are assigned, they can serve as good probes to monitor the behaviors of these groups in the electron transfer reactions. However, the assignment is not easy, because many signals are scattered due to the paramagnetic effects of irons and the ring current of porphyrins. We have carried out the assignment of phenylalanine signals through the specific deuteration of these residues in cytochrome c_3. Since the conditions for the culture in the minimal medium were not yet established, we have to determine those conditions first.

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Materals and Methods

Deuteration of phenylalanine

The deuteration of all the aromatic protons of phenylalanine was carried out according to reported methods (Matthews *et al.*, 1977) with minor modifications. In order to remove exchangeable protons, 10 g of phenylalanine was dissolved in a mixture of 30 ml of ^2H_2O (^2H; 99.9%, Showadenko) and 10 ml of 6 N ^2HCl (^2H; 99%, CIL). The solvent was removed by a rotary evaporator. The sample was further vacuum-dried. Then, it was dissolved in 80 g of 85% (w/w) $^2H_2SO_4$ (originally 97%, ^2H; 99%, CIL). The solution was incubated at 50℃ for 72 h. After dilution to 1l by distilled water, sulfate ions were removed by adding BaOH. Deuterated phenylalanine was repeated twice. The total yield of the deuterated phenylalanine was 87%. Judged from the 200 MHz NMR spectrum, 98% of the aromatic protons were deuterated through this procedure.

Culture and purification

Desulfovibrio vulgaris Miyazaki F was cultured in the C medium proposed by Postgate (1984), unless otherwise mentioned. Cytochrome c_3 was purified according

Table 1. A minimal medium for *D. vulgaris* Miyazaki F (per liter)[a]

KH_2PO_4	0.5 g	Asp	0.4 g
NH_4Cl	1.0 g	Cys	25.0 mg
Na_2SO_4	4.5 g	Gln	0.1 g
$CaCl_2 \cdot 6H_2O$	0.06 g	Glu	1.0 g
$MgSO_4 \cdot 7H_2O$	0.06 g	Gly	0.1 g
Sodium lactate	6.0 g	Ile	0.3 g
$FeSO_4 \cdot 7H_2O$	4.0 mg	Leu	0.4 g
Sodium citrate$\cdot 2H_2O$	0.3 g	Lys	0.45 g
Selenite solution[b]*	1.0 ml	Met	0.1 g
Trace elements[c]*	1.0 ml	Phe	0.2 g
Vitamines[d]*	0.1 ml	Pro	0.4 g
Growth stimulants[e]*	0.1 ml	Ser	0.3 g
Other acid[f]*	0.1 ml	Thr	0.2 g
Amino acid mixture		Val	0.3 g
Ala	0.2 g	His	0.15 g
Arg	0.2 g	Tyr	0.4 g
Asn	0.04 g	Trp	0.1 g

[a] Sterilize by autoclaving, componets marked* added aseptically later.

[b] From autoclaved stock of Na_2SeO_3 3 mg+NaOH 0.5 g/l.

[c] From autoclaved stock of $FeCl_2 \cdot 4H_2O$, 1.5 g; H_3BO_3, 60 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; $CoCl_2 \cdot 6H_2O$, 120 mg; $ZnCl_2$, 70 mg; $NiCl \cdot 6H_2O$, 25 mg; $CuCl_2 \cdot 2H_2O$, 15 mg; $NaMoO_4 \cdot 2H_2O$, 25 mg/l.

[d] From filter-sterilized stock of biotin, 1 mg; *p*-aminobenzoic acid, 5 mg; vitamin B_{12}, 5 mg; thiamine, 10 mg/ml.

[e] From autoclaved stock of isobutyric acid, varic acid, 2-methylbutyric acid, 3-methylbutric acid, 0.5 g of each; caproic acid, 0.2 g; succinic acid, 0.6 g/100 ml, NaOH to pH 9.

[f] From autoclaved stock including; Na acetate$\cdot 3H_2O$, 20 g, propionic acid, 7 g, *n*-butyric acid, 8 g, benzoic acid, 5 g, *n*-palmitic acid, 5 g/100 ml, NaOH to pH 9.

to the procedure reported previously (Park *et al.*, 1991). The purity index (A_{552}(red)/A_{280}(ox)) of the purified sample was higher than 2.9. The purity was also confirmed by SDS polyacrylamide gel electrophoresis.

NMR measurments

^{1}H-NMR spectra 500 MHz was measured with JEOL GX500 NMR spectrometers. Cytochrome c_3 was dissolved in a 30 mM phosphate buffer (p^{2}H 7.0) solution. Two-dimensional COSY spectrum was measured with 512×2048 points and 25000 Hz spectral width at a protein concentration of 4 mM. Chemical shift was shown relative to an internal standard of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). ^{2}H-NMR spectra at 55.3 MHz were obtained with a Bruker WM 360 wb NMR spectrometer.

Results and Discussion

A minimal medium for the growth of sulfate reducing bacteria

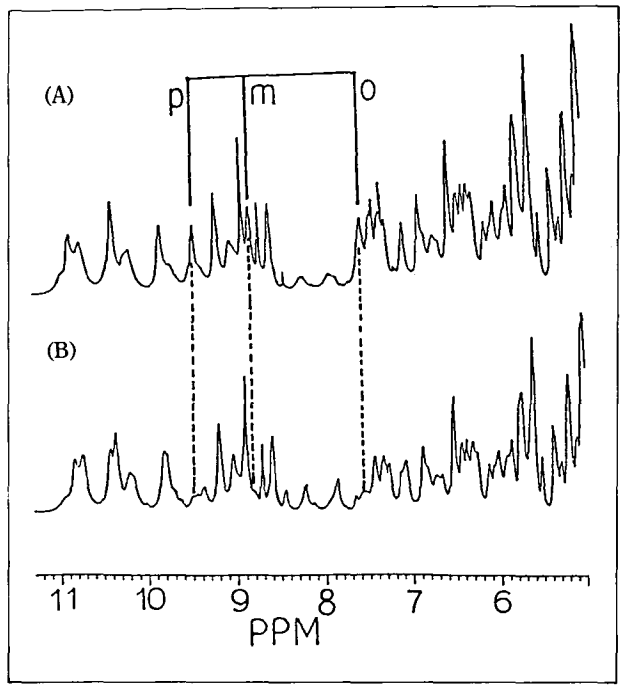

Fig. 1. 500 MHz ^{1}H-NMR spectra of ferricytochrome c_3 at 25℃. A: normal, B: specifically deuterated at phenylalanine residues. The assignments of the ortho, meta and para ring protons of phenylalanine are given as o, m and p, respectively, in the figure.

It was found that *D. vulgaris* Miyazaki F (*Dv*MF) can be cultured in a minimal medium as shown in Table 1.

The composition of this medium was arranged by referring to media C and G proposed by Postgate and Pfenning *et al.*, respectively (Postgate, 1984), and a minimal medium for *E. coli* (Matthews *et al.*, 1977). A better yield was obtained in the presence of 0.05 g/l of yeast extract. The yield was about 70% of that in the culture with medium C. This minimal medium was used for the specific deuteration of cytochrome c_3. In the deuteration of this work, 0.2 and 0.8 g/l of deuterated phenylalanine was used in place of the nondeuterated one.

^{1}H-NMR spectra of specifically deuterated ferricytochrome c_3

A 500 MHz ^{1}H-NMR spectrum of ferricytochrome c_3 specifically deuterated at phenylalanine residues (Dphe-cyt c_3) in the region from 5 to 11 ppm at 25℃ is presented in Fig. 1 in comparison with the spectrum of the nondeuterated one.

Fig. 1 In the former spectrum, signals at 7.6, 8.8, 9.5 ppm were not observed. Therefore, these signals can be assigned to aromatic protons of phenylalnine residues. From the integrated intensity, the extent of deuteration was estimated to be about 85%.

*Dv*MF cytochrome c_3 has two phenylalnine residues, namely, Phe20 and Phe76. They should give rise to

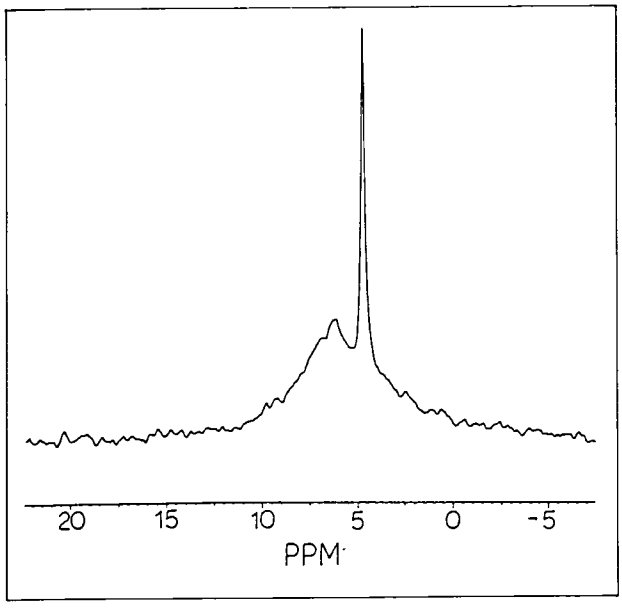

Fig. 2. 55.3 MHz ^{2}H-NMR spectrum of ferricytochrome c_3 specifically deuterated at phenylalanine residues at 30℃. The sharp signal is due to water.

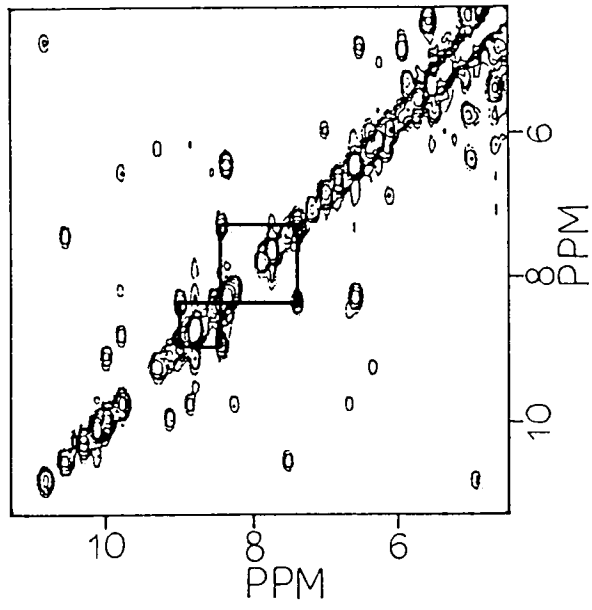

Fig. 3. 500 MHz two-dimensional COSY spectrum of ferricytochrome c_3 in the aromatic region at 50℃. The connectivity of phenylalanine signals is shown by solid line.

more than six signals. The result of Fig. 1 showed that the signals of only one phenylalnine residues appeared in this region. Since the signal at 9.5 ppm has the intensity of a single proton, it can be assigned to 4 (or para)-H of the aromatic ring. We could not find the signals of the other phenylalanine residue in the entire spectrum. A ^{2}H-NMR spectrum of *D*Phe-cyt c_3 at 55.3 MHz is shown in Fig. 2.

A broad signal with a peak at 6.5 ppm was observed in addition to the water signal. The weight-averaged chemical shift of the three phenylalanine signals in Fig. 1 was about 8.5 ppm. The fact suggests that the signals due to the other phenylalanine residue should be located in the higher field region than the observed ones. The assigned phenylalanine signals were also observed in a 2D COSY spectrum at 50℃ shown in Fig. 3.

It is clear from this spectrum that the central signal (8.8 ppm at 25℃) is coupled with the other two. Therefore, the signals at 7.6 and 8.8 ppm could be assigned to 2.6 (or orther) and 3.5 (or meta) ring protons, respectively. This was also confirmed by the 1D NOE (nuclear Overhauser effect) experiment (data not shown). Since only three signals were observed for one phenylalanine residue, the flip rate of this aromatic ring is faster than the NMR time scale. On the other hand, the motion of the other phenylalanine residue is expected to be close to the NMR time scale, leading to the broadening of the signals. This is also supported by the fact that the contribution of this residue appeared in the spectrum at 55.3 MHz, as can be seen in Fig. 2.

In summary, growth conditions of *Desulfovibrio vulgaris* Miyazaki F in a minimal medium were established in this work. The yield of the cells and the efficiency of the specific deuteration of cytochrome c_3 were satisfactory at least for phenylalanine. This system can be used not only for the deuteration of other amino acid residues but also for the labeling of proteins with other stable isotopes.

*Dv*MF cytochrome c_3 has two phenylalanine residues, namely, Phe20 and Phe76. Phe20 is only residues, which is conserved in all amino acid sequencs of cytochrome c_3 determined so far. In crystal structure, it intervences heme III and IV, orienting its aromatic ring parallel to the porphyrin ring of heme III and to the imidazole ring of His25, a ligand of heme IV (Higuchi *et al.*, 1984). In contrast, Phe76 is located in the position close to the surface of the protein. Thus, the ring of Phe76 is expected to be more mobile than that of Phe20. It leads to a tentative assignment of the observed signals to Phe76.

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