# $^1$H NMR Studies of the Interaction between Cytochrome $c_3$ and Ferredoxin I from *D. vulgaris* Miyazaki F

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*Received January 30, 1999*

Heme assignment of the $^1$H NMR spectrum of cytochrome $c_3$ of *D. vulgaris* Miyazaki F was established [Reference: 12, 13]. The major reduction of the heme turned out to take place in the other of heme 4, 1, 2 and 3 (in the sequential numbering). The Hemes with the smallest and greatest solvent accessibility were reduced at the highest and lowest potentials in average, respectively. A cooperation interheme interaction was attributed to a pait of the closest hemes, namely, hemes 1 and 2. This assignment can provide the physicochemical bases for the elucidation of electron transfer of this protein.

## Introduction

Cytochrome $c_3$ and ferredoxin I are multi-redoxsite proteins of a sulfate-reducing bacteria, *Desulfovibrio vulgaris* Miyazaki F (*D.v.*MF). The former and latter have four hemes and two iron-sulfur clusters ([4Fe-4S] and [3Fe-4S]), respectively, in a single polypeptide unit.[1,2] Their molecular weights are 14000 and 6000, respectively.[1,3] They are involved in the electron transport system in the metabolism of sulfur compounds and hydrogen.[4] Although they can be redox partners in vitro,[3] their detailed roles in vivo are not yet clarified. The redox potentials of cytochrome $c_3$ are very low in comparison with other cytochromes.[5] Thus, this was classified to class III cytochromes.[6] The crystal structures of cytochrome $c_3$ from *D. vulgaris* Miyazaki F.[7] and *D. desulfricans* Norway[8] have been reported at 0.18 and 0.25 nm resolution, respectively. Although they provided the important information on the cytochrome $c_3$ structure, the roles of four hemes and the reasonings for the low redox potentials were not yet elucidated. Nuclear magnetic resonance (NMR) is one of the powerful methods to investigate these problems. Extensive NMR works of cytochrome $c_3$ were carried out in this decade.[9,10] The macroscopic and microscopic redox potentials of *D.v.*MF Cytochrome $c_3$ were successfully analyzed by $^1$H NMR in combination with spectroelectrochemical method.[10,11] It was shown that each heme has an unique redox potential, which also depends on oxidation states. Site-specific heme assignment of the $^1$H NMR spectrum of cytochrome $c_3$ of *D. vulgaris* Miyazaki F was established by Park et al.[12] In this work, on the basis of heme assignment, the interaction of cytochrome $c_3$ with ferredoxin I was investigated by NMR. To simplify the heme numbering of cytochrome $c_3$, we adopted the sequential heme numbering[14,15,16] following other research groups. Hemes 1, 2, 3, and 4 in the sequential numbering corresponds to hemes 3, 2, 4 and 1 in the reported crystal structure,[7] respectively. In the case of the order of reduction, however, we would like to use heme $i'$ (abbreviation, $h_{i'}$) instead of $h_{i'}$,[14] in order to make the notation for the microscopic redox potentials simpler and clearer. The IUPAC-IUB nomenclature[17] is used for the heme carbons and protons in this paper.

## Material and Methods

Cytochrome $c_3$ was purified from *Desulfovibrio vulgaris* Miyazaki F (*D.v.*MF) cells according to a modified method originally proposed by Yagi *et al.*[18] The wet cells suspended in a two-volume of 30 mM phosphate buffer, pH 7.0 in the presence of deoxyribonuclease I (Sigma, three hundred-thousandth of the wet cell weight) at 4 °C. After the sonication (at 70W and 4 °C for 45 min), the solution was centrifuged at 40000 rpm for 2 hr with an ultracentrifuge Hitachi himac CP70 (rotor; RP42). A certain amount of streptomycin sulfate was added to the supernatant to remove polynucleotides. After centrifugation at 20000 rpm for 30 min (the same rotor), the supernatant was dialyzed against 10 mM phosphate buffer, pH 7.0, then applied to an Amberlite CG-50 Type I column (Nacl 0-1.0 M). The cytochrome $c_3$ fraction was desalted and concentrated, then applied to the same column (Nacl 0-0.5 M), followed by the purification on FPLC (Parmacia) with Mono S and Superose columns. Temperature was kept at 4 °C througout the purification process. The purity index ($A_{552}$(red)/$A_{280}$(ox)) of the purified sample was greater than 3.0. The purity was also confirmed by SDS-polyacrylamide gel electrophoresis. Ferredoxin I was purified from *D.v.*MF cells according to the reported method.[2] In the handling of Ferredoxin I, a special care was taken not to expose it to oxygen. For NMR measurements, the proteins were dissolved in 30 mM phosphate buffer (p$^2$H 7.0) solutions. Deuteration of exchangeable protons of cytochrome $c_3$ was carried out by lyophilization and dissolution in a deuterated buffer solution. Ferredoxin I was washed with a deuterated buffer solution repeatedly on membrane filter (YM5) under nitrogen gas flow. 400 MHz $^1$H NMR spectra were measured with a Bruker AM400 NMR spectrometer unless otherwise mentioned. Chemical shifts are

presented in parts per million relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). In the case of ferredoxin titration, aliquot of a 0.73 mM ferredoxin I solution was added to 0.4 ml of a 0.48 mM cytochrome $c_3$ solution and the spectrum was measured at 25 °C. The ionic strength was 53 mM (30 mM phosphate buffer, pH 7.0). The protein concentration was determined using the absorption coefficients 120 $mM^{-1}$ $cm^{-1}$ for cytochrome $c_3$[5] and 35 $mM^{-1}$ $cm^{-1}$ (per monomer) for ferredoxin I.[4]

## Results and Discussion

The assignment of 16 heme methyl (designated as A-P) signals of $^1H$ NMR spectrum of ferricytochrome $c_3$ from *D. vulgaris* Miyazaki F has been performed.[12]

$^1H$ NMR spectra of cytochrome $c_3$ mixed with ferredoxin I at a variety of molar ratio was presented in Figure 1.

The heme methyl signals of cytochrome $c_3$ can be clearly monitored even in the presence of ferredoxin I. The chemical shift changes of heme methyl signals of cytochrome $c_3$ are plotted as a function of molar ratio of ferredoxin I to cytochrome $c_3$ in Figure 2. The largest and second largest chemical shift changes were observed for signals H and A, respectively. Otherwise, the change was smaller than 0.2 ppm. Since, the signals of heme 1 changed little, they were not included in Figure 2.

The heme assignment mentioned[12] can be applied to the analysis of the interaction of cytochrome $c_3$ with ferredoxin I. It is interesting to see the interaction mechanism between multi-redoxsite proteins. On addition of ferredoxin I, the largest change of the chemical shift was observed for signal H and A of cytochrome $c_3$. Both of them belong to heme I and are exposed to solvent. This fact strongly suggests that the interaction site of cytochrome $c_3$ is heme I. The data for signal H in Figure 2 were analyzed by nonlinear-least-square fitting on two cytochrome $c_3$ per monomer of ferredoxin I gave a good fitting as shown in Figure 3.

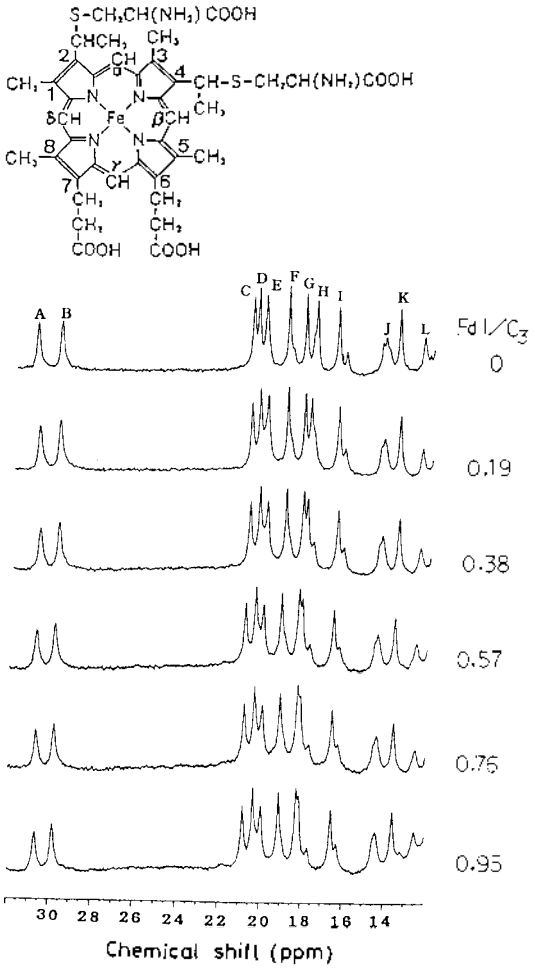


**Figure 1.** NMR spectra of ferricytochrome $c_3$ titrated by ferredoxin I at 25 °C. The original concentrations of cytocrome $c_3$ and ferredoxin I were 0.48 mM and 0.73 mM (in 30 mM phosphate buffer, $p^2H$ 7.0), respectively. The ratio of ferredoxin I (monomer) per cytochrome $c_3$ is shown on the right of each spectrum. The heme methyl signals were labeled alphabetically. The chemical structure of a c-type heme and the labels of the prophyrin carbons are given on top.

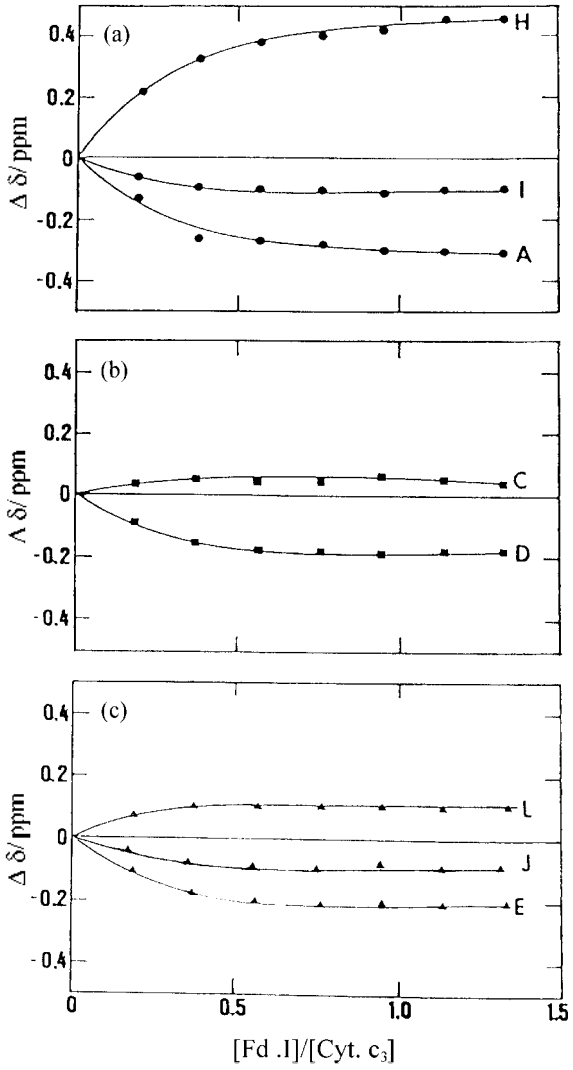


**Figure 2.** The chemical shift changes of heme methyl resonances of ferricytochrome $c_3$ as a function of ferredoxin I (monomer) per cytochrome $c_3$. (a), (b) and (c); resonances belong to hemes 4, 2 and 3, respectively.

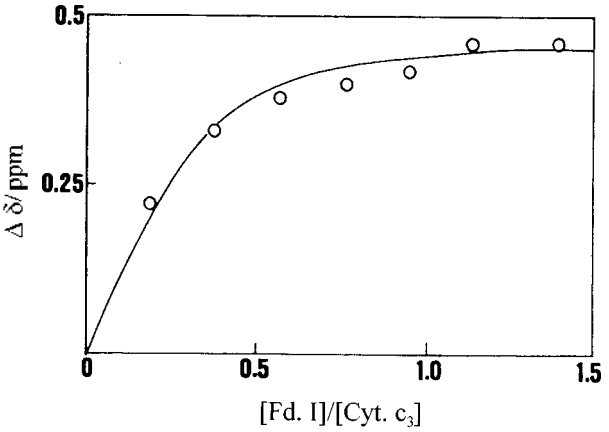


**Figure 3.** Simulation of the titration curve of signal H of ferricytochrome $c_3$. The solid line represents the simulation curve with the stoichiometry of two cytochrome $c_3$ per one ferredoxin I (monomer) and association constant of $10^8$ $M^{-2}$.

The association constant was estimated to be $10^8$ $M^{-2}$ at the ionic strength of 53 mM and at 25 °C. Although only one redox site of cytochrome $c_3$ is mainly involved in, all the redox sites are used for binding in the case of ferredoxin I.

Guerlesquin *et al.* investigated the interaction between cytochrome $c_3$ and ferredoxin I from *D. desulfuricans* Norway by NMR[19] and calorimeter.[20] The stoichiometry of the complex was one molecule of cytochrome $c_3$ per monomer of ferredoxin I. Since ferredoxin I from *D. desulfuricans* Norway has only one iron-sulfur cluster, the stoichiometry is four hemes per one iron-sulfur cluster. In contrast, ferredoxin I from *D.v.*MF has two iron-sulfur cluster.[2] Therefore, although the molecular stoichiometry in the case *D.v.*MF was different from that of *D. desulfuricans* Norway, its redoxsite stoichiometry was also four hemes per one iron-sulfur cluster. Since the redox potentials of two iron-sulfur clusters of ferredoxin I were reported to be higher and lower than that of heme 4,[3] they could be the pathway of electron flow for the forward and backward in the in vitro electron exchanges. Guerlesquin et al. obtained association constant of $10^4$ $M^{-1}$ by NMR and $10^6$ $M^{-1}$ by calorimetry. They attributed the origin of the difference in the association constant to the difference in protein concentration.[20] The former is in good agreement with ours provided that two binding sites of *D.v.*MF ferredoxin I have the same association constant. Judging from the chemical shift change, the major interaction site of cytochrome $c_3$ is heme 4 for both binding sites. In the case of *D. desulfuricans* Norway, the chemical shifts of hemes with the highest and second highest redox potentials changed to the similar extent on the complex formation in spite of one to one stoichiometry.[19] In the crystal structure of cytochrome $c_3$ from *D.v.*MF, heme 4 is surrounded by the highest density of positive charges, as in the case of *D. desulfuricans* Norway. In the model study on the complex formation cytochrome $c_3$ with ferredoxin from *D. desulfuricans* Norway, Cambillau et al. specularted that heme 4 (corresponding to heme 4 in *D.v.*MF) is the most favorable interaction site.[21] Actually, weakening of the binding was observed with the increase of the ionic strenth in our case (data not shown). However, Guerlesquin *et al.* indicated that the interaction is essentially hydrophobic in spite of the involvement of electrostatic interactions, because it is an entropy-driven reaction.[20] The interactions of cytochrome $c_3$ with acidic proteins such as flavodoxin and rubredoxin were also investigated experimentally[22] and on the computer graphics.[23] These proteins were also anticipated to interact with heme 4 by interacting with the amino acid residues surrounding it.

**Acknowledgment.** This work was supported mainly by the Matching Fund Programs of Research Institute for Basic Science, Pusan National University, Korea, 1999, Project No. RIBS-PNU-99-303. Authors thank the KOSEF (CBM of POSTECH) for the partial financial support.

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