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Full assignment of heme redox potentials of cytochrome c_3 of *D. vulgaris* Miyazaki F by ^{1}H-NMR

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Site-specific heme assignment of the ^{1}H-NMR spectrum of cytochrome c_3 of *D. vulgaris* Miyazaki F, a tetraheme protein, was established. The major reduction of the heme turned out to take place in the order of hemes I, III, IV and II (numbering in the crystal structure). The hemes with the smallest and greatest solvent accessibility were reduced at the highest and lowest potentials on average, respectively. A cooperative interheme interaction was attributed to a pair of the closest hemes, namely, hemes III and IV. This assignment can provide the physicochemical basis for the elucidation of electron transfer of this protein.

Cytochrome c_3; Tetraheme protein; Redox potentials of a tetraheme protein; ^{1}H-NMR; Assignment of redox potential

1. INTRODUCTION

Cytochrome c_3 is an unique class of heme proteins which contain 4 hemes in a single polypeptide and show very low redox potentials [1]. Crystal structures of cytochrome c_3 from *Desulfovibrio desulfuricans* Norway (*D.d*N) and *Desulfovibrio vulgaris* Miyazaki F (*D.v.*MF) 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. Macroscopic and microscopic redox potentials were determined for a series of cytochromes 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 *D.v.*MF cytochrome c_3 using electron spin resonance (ESR) [7]. Fan et al. [6] assigned the highest and third highest redox potentials of the same protein by the use of nuclear magnetic resonance (NMR), which contradicted the assignment by ESR. So far, there is no full assignment for this protein. The assignment was also in conflict between chemical modification [8] and ESR [9] for *D.d.*N cytochrome c_3. A full assignment of *D.v.*MF cytochrome c_3 was performed by NMR in combination with isotope labeling in this work. The result agreed with the partial assignment by Fan et al.

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2. MATERIALS AND METHODS

Desulfovibrio vulgaris Miyazaki F was cultured in medium C [1] and in a minimal medium [10] to obtain nondeuterated and deuterated cytochrome c_3, respectively. In the latter case, ϵCH_3 of methionine was specifically deuterated by replacing methionine of the minimal medium with L-methionine-methyl-d_3 (^{2}H; 98%, CIL). Cytochrome c_3 was purified according to the reported procedure [10]. The purity index (A_{552}(red)/A_{280}(ox)) of the final sample was over 3.0. The purity was also confirmed by SDS-polyacrylamide gel electrophoresis. 400 MHz ^{1}H-NMR spectra of 2.7 mM cytochrome c_3 in 30 mM phosphate buffer (p^{2}H 7.0) were measured at 30°C with a Bruker AM-400 NMR spectrometer. Chemical shifts are presented in parts per million relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The nuclear Overhauser effect (NOE) experiments were performed with 0.5 s pre-irradiation and the accumulation of 4800 transients. The difference spectrum was obtained by subtracting the on-resonance free induction decay (FID) from the off-resonance FID. Two-dimensional (2D) TOCSY (HOHAHA) spectra were measured at 30°C with the data size of 512 × 2048, spectral width of 8064 Hz and mixing time of 26.6 ms. 2D-COSY spectra were measured at 30 and 50°C with the data size of 512 × 2048 and spectral width of 13900 Hz. The p^{2}H values reported in this paper are pH-meter readings uncorrected for isotope effects.

3. RESULTS AND DISCUSSION

A 400 MHz ^{1}H-NMR spectrum of ferricytochrome c_3 is shown at the top of Fig. 1. Thirteen heme methyl signals were assigned by Fan et al. [6] and they were labeled alphabetically in the figure. A strong nuclear Overhauser effect (NOE) was observed at −0.29 ppm on irradiation at signal I (Fig. 1c). The same NOE signal appeared on irradiation at signal G (Fig. 1d). This signal showed three-proton intensity. This signal has no cross peak in 2D-TOCSY and COSY spectra. Therefore, this may be an isolated methyl proton spin. Since an isolated methyl spin system is either ϵCH_3 of

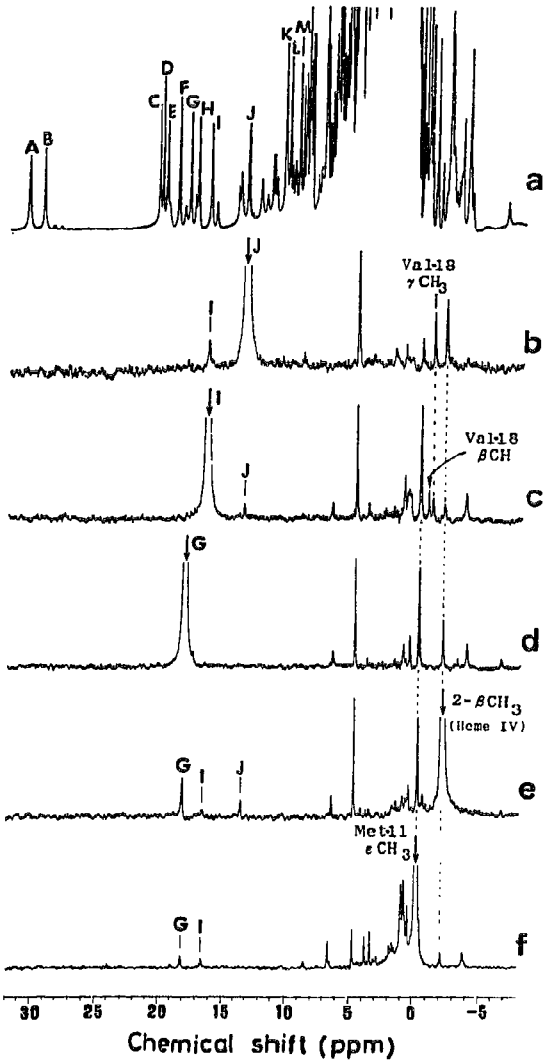

Fig. 1. 400 MHz ^{1}H-NMR spectra of ferricytochrome c_3 from *D. vulgaris* Miyazaki F in 30 mM phosphate buffer (p^2H 7.0) at 30°C. The heme methyl signals are labeled alphabetically. (a) A normal spectrum. (b–f) NOE difference spectra. The arrows indicate the irradiated positions. The signal at 4.73 ppm comes from H^2HO. The assignment of the signals is given in the text.

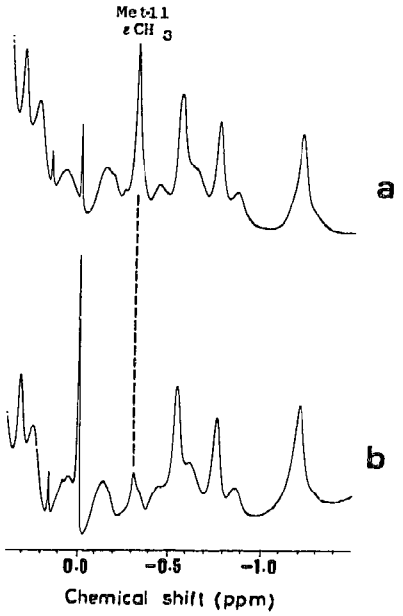

Fig. 2. 400 MHz ^{1}H-NMR spectra of nondeuterated (a) and deuterated (b) ferricytochrome c_3 at 30°C. Cytochrome c_3 was specifically deuterated at the ϵCH_3 of methionine residues. The assignment is given in the text.

methionine or a heme methyl group, the cytochrome c_3 was specifically deuterated by deuterated methionine. Actually, the signal of interest almost disappeared on the deuteration as shown in Fig. 2. The NOE signals in Fig. 1 also disappeared when the deuterated sample was used. The NOE signal at −0.29 ppm could be attributed to ϵCH_3 of a methionine residue through the inspection of the results. The chemical shift of this signal is unusually high in comparison with that of a normal methionine. This is consistent with the close proximity of this methyl group to porphyrin rings. In the crystal structure, only the ϵCH_3 of Met-11 was found to be close to heme methyl groups. It is located at a distance of 4.10 and 3.89 Å from the 5-CH_3 of heme I and the 5-CH_3 of heme III, respectively, in terms of carbon distance. Therefore, signals I and G can be assigned to the 5-CH_3 of hemes I and III. The heme numbering is due to Higuchi et al. [3].

On irradiation at signal J, a strong NOE was observed at −2.11 ppm (Fig. 1b). The same NOE signal appeared on irradiation at signals I and G (Fig. 1c and d). This signal has a three-proton intensity. The relevant part of TOCSY spectrum is presented in Fig. 3. This chemical group should have A_3X_n spins as a part of its coupled spin system. There was no amino acid residue of this type, the side chain of which is close to the 5-CH_3 groups of hemes I and III (signals I and G) in the crystal structure. Besides the amino acid residues, this type of spin system can be found in the heme side chain (βCH_3-αCH) in the thioether bridge. Actually, only one connectivity was observed also in 2D-COSY spectra. The inspection of the crystal structure showed that the 2-βCH_3 of heme IV is close to the 5-CH_3 of hemes I and III. Their carbon distances were 4.78 and 3.60 Å, respectively. Thus, the NOE signal at −2.11 ppm can be assigned to the 2-βCH_3 of heme IV. This assignment was further supported by the NOE with the ϵCH_3 of Met-11 shown in Fig. 1e and f, because the carbon distance between the 2-βCH_3 of heme IV and ϵCH_3 of Met-11 is 4.29 Å in the crystal structure. Now, signal J can be ascribed to the 1-CH_3 of heme IV because of its proximity to the 2-βCH_3 of the same heme. On the basis of the NOE between signals I and J, these signals were attributed to the 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively, by Fan et al. [6]. This assignment was supported by the NOE among the signals I, J and side chain of Val-18 [11]. They can be seen in Fig. 1b and c. The assignment of

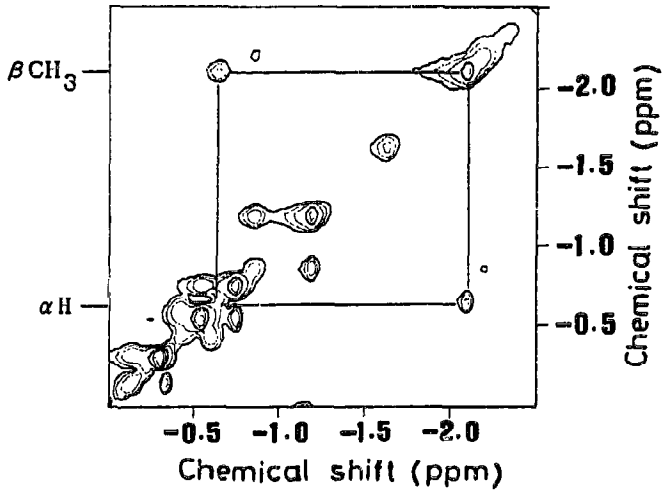

Fig. 3. Section of a TOCSY (HOHAHA) spectrum of ferricytochrome c_3 at 30°C, showing the J-connectivities for the heme side chain in the thioether bridge.

signals I and J is consistent with the assignments so far mentioned in this work. Since signal G was due to the 5-CH_3 of either heme I or III, and signal I turned out to be the 5-CH_3 of heme I, signal G can be now ascribed to the 5-CH_3 of heme III. The NOE network and the corresponding carbon distances in the crystal structure are summarized in Fig. 4. The excellent matching in Fig. 4 confirms the reliability of the assignment and suggests that the crystal structure is maintained in solution to a great extent.

Signals I, G and J belong to the hemes mainly reduced at the first, second and third reduction steps, respectively [6]. Therefore, it can be concluded that hemes I, III, IV and II in the crystal structure are mainly reduced in this order. In other words, they can be assigned to hemes 1, 2, 3, and 4 (numbering due to the order of major reduction [6]), respectively. This is not compatible with the assignment by Gayda et al. [7]. On the basis of this assignment, the microscopic redox potentials established by Fan et al. [6] can be attributed to the individual hemes in the crystal structure. Thus, this full assignment enables us to investigate the structural factors which determine the redox potential of each heme in a tetraheme protein. For example, the solvent accessibility is the smallest for heme I and increases in the order of hemes III, IV and II [3]. This well corresponds to the order of the major reduction of the hemes. A cooperative interheme interaction was shown for the pair of hemes 2 and 3 by Fan et al. [6]. This pair can be attributed to hemes III and IV in the crystal structure on the basis of our assignment. Their inter-iron distance is 11 Å and is the shortest in this protein. Furthermore, Phe-20, which is conserved in most cytochromes c_3 so far sequenced, is intervening these hemes in a way almost parallel to planes of heme III and the imidazole ring ligated to heme IV. The cooperative interheme interaction might be connected with this specific conformation. However, this is the subject for future investigation.

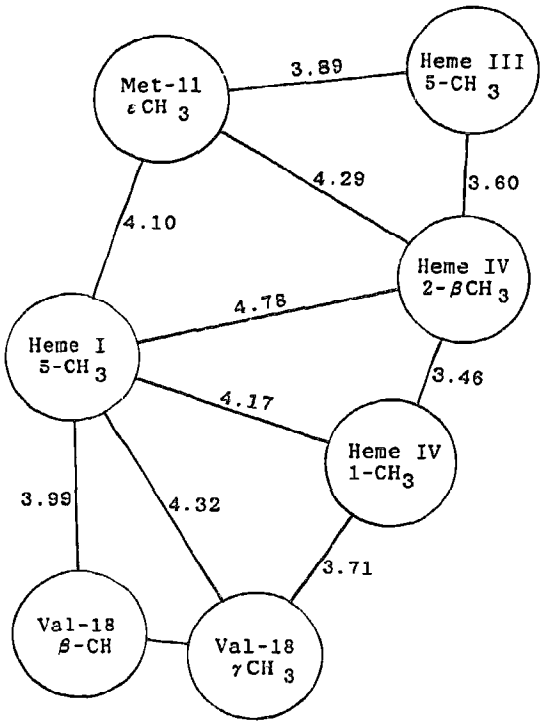

Fig. 4. Correlation between the NOE network (solid lines) and the intercarbon distances (Å) in the crystal structure. The assignment is given in the text.

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