Journal of Biomolecular NMR, 1 (1991) 271–282
ESCOM

J-Bio NMR 018

^{1}H NMR studies on ferricytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F and its interaction with ferredoxin I

Jang-Su Park[a], Katsuhiro Kano[a], Yukio Morimoto[b], Yoshiki Higuchi[b], Noritake Yasuoka[b], Mari Ogata[c], Katsumi Niki[a] and Hideo Akutsu[a,*]

[a]*Department of Physical Chemistry, Faculty of Engineering, Yokohama National University, Hodogaya-ku, Yokohama 240, Japan*

[b]*Faculty of Science, Himeji Institute of Technology, Shosha-2167, Himeji, Hyogo 671-22, Japan*

[c]*Department of Chemistry, Shizuoka University, Oya, Shizuoka 422, Japan*

Received 15 February 1991
Accepted 3 April 1991

Keywords: Cytochrome c_3; Ferredoxin I; ^{1}H NMR; Assignment of heme signals; Inter-redoxsite interaction

SUMMARY

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** (1990) 2257–2263). The site-specific heme assignments were also supported by NOE between the methyl groups of these hemes and the side chain of Val^{18}. All the results contradicted the heme assignments for *D.v.* MF cytochrome c_3 made on the basis of electron spin resonance (Gayda et al. (1987) *FEBS Lett.*, **217** 57–61). Based on these assignments, the interaction of cytochrome c_3 with *D.v.* MF ferredoxin I was investigated by NMR. The major interaction site of cytochrome c_3 was identified as the heme with the highest redox potential, which is surrounded by the highest density of positive charges. The stoichiometry and association constant were two cytochrome c_3 molecules per monomer of ferredoxin I and 10^8 M^{-2} (at 53 mM ionic strength and 25°C), respectively.

INTRODUCTION

Cytochrome c_3 and ferredoxin I are multi-redox-site proteins of a sulfate-reducing bacterium, *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, per single polypeptide unit (Shinkai et al., 1980; Okawara et al., 1988). Their molecular weights are 14000 and 6000, respectively (Shinkai et

* To whom correspondence should be addressed.

al., 1980; Ogata et al., 1988). They are involved in the electron transport system in the metabolism of sulfur compounds and hydrogen (Postgate, 1984). Although they can be redox partners in vitro (Ogata et al., 1988), their detailed roles in vivo have not yet been clarified. The redox potentials of cytochrome c_3 are very low in comparison with those of other cytochromes (Niki et al., 1987). Thus, it was classified as a class III cytochrome (Pettigrew and Moore, 1987). The crystal structures of cytochromes c_3 from *D.v.* MF (Higuchi et al., 1984) and *D. desulfuricans* Norway (Pierrot et al., 1982) have been reported at 0.18 and 0.25 nm resolution, respectively. Although they provided important information on the cytochrome c_3 structure, the roles of the four hemes and the reasons for the low redox potentials were not elucidated. Nuclear magnetic resonance (NMR) is one of the powerful methods for investigating these problems. Extensive NMR studies on cytochrome c_3 have been carried out in the last decade (Santos et al., 1984; Guerlesquin et al., 1985a; Fan et al., 1990a, and references therein). The macroscopic and microscopic redox potentials of *D.v.* MF cytochrome c_3 were successfully determined by ^{1}H NMR in combination with a spectroelectrochemical method (Fan et al., 1990a,b). It was shown that the four hemes have different redox potentials, and that the redox potential of one heme is affected by the oxidation states of the other hemes. The correlations between the heme methyl signals and two particular hemes in the crystal structure have been established (Fan et al., 1990a). The redox potentials of cytochrome c_3 have also been investigated by electron spin resonance (ESR; Gayda et al., 1987; Benosman et al., 1989). Their results contradicted those obtained on NMR measurement in some aspects. In particular, the site-specific assignments of hemes were different from those determined by NMR (Gayda et al., 1987; Fan et al., 1990a). This is a very important point as to the elucidation of the physicochemical properties of cytochrome c_3 on the basis of its molecular conformation. Consequently, we have carried out a detailed assignment of the heme methyl, propionate and related signals in this work. Our results confirmed the heme assignments determined by NMR. In the light of these assignments, the interaction of cytochrome c_3 with ferredoxin I was also investigated.

MATERIALS AND METHODS

Cytochrome c_3 was purified from *D.v.* MF cells according to a modified method originally proposed by Yagi and Maruyama (1971). The wet cells were suspended in two volumes of 30 mM phosphate buffer, pH 7.0, containing deoxyribonuclease I (Sigma; 3×10^{-5} of the wet cell weight) at 4°C. After sonication at 70W and 4°C for 45 min, the solution was centrifuged at 40000 rpm for 2 h in a Hitachi Himac CP70 ultracentrifuge (rotor, RP42). A certain amount of streptomycin sulfate was added to the supernatant to remove polynucleotides. After further centrifugation at 20000 rpm for 30 min (the same rotor), the supernatant was dialyzed against 10 mM phosphate buffer, pH 7.0, and then applied to an Amberlite CG-50 Type I column (NaCl, 0 – 1.0 M). The cytochrome c_3 fraction was desalted and concentrated, and then applied to the same column (NaCl, 0 – 0.5 M), followed by purification by FPLC (Pharmacia) on MonoS and Superose columns. The temperature was maintained at 4°C throughout 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 method reported by Okawara et al. (1988). In the handling of ferredoxin I, special care was taken not to expose it to oxygen. For NMR measurements, the proteins were dissolved in 30

mM phosphate buffer (p^2H 7.0). Deuteration of exchangeable protons of cytochrome c_3 was carried out by lyophilization and dissolution in a deuterated buffer solution. Ferredoxin I was repeatedly washed with the deuterated buffer solution on a membrane filter (YM5) under a flow of nitrogen gas.

400 MHz ^1H NMR spectra were obtained at 30°C with a Bruker AM400 NMR spectrometer. Chemical shifts are presented in parts per million relative to the internal standard, 2,2-dimethylsilapentane-5-sulfonate (DSS). In the nuclear Overhauser effect (NOE) experiments, 16 transients were accumulated under on- and off-resonance irradiation for 0.5 s, alternately. In total, 4800 transients were accumulated for each case, unless stated otherwise. An NOE difference spectrum was obtained from these free-induction decays. Two-dimensional DQFCOSY spectra were obtained with a data size of 512 × 2048 and spectral width of 25 000 Hz. Two-dimensional TOCSY (HOHAHA) spectra were obtained with a data size of 512 × 2048, spectral width of 8064 Hz and mixing time of 26.6 ms. Sixty-four transients were accumulated. The protein concentration was 2.7 mM. In the case of ferredoxin titration, an 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 then 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 absorption coefficients of 120 mM^{-1} cm^{-1} for cytochrome c_3 (Niki et al., unpublished data) and 35 $mM^{-1}cm^{-1}$ (per monomer) for ferredoxin I (Ogata et al., 1988).

RESULTS

Assignments of heme proton signals

A 400 MHz ^1H NMR spectrum of ferricytochrome c_3 is shown in Fig. 1, along with the chemical structure of a c-type heme. As reported by Fan et al. (1990a), 13 heme methyl signals can be observed separately in the downfield region. They are labeled alphabetically from the downfield.

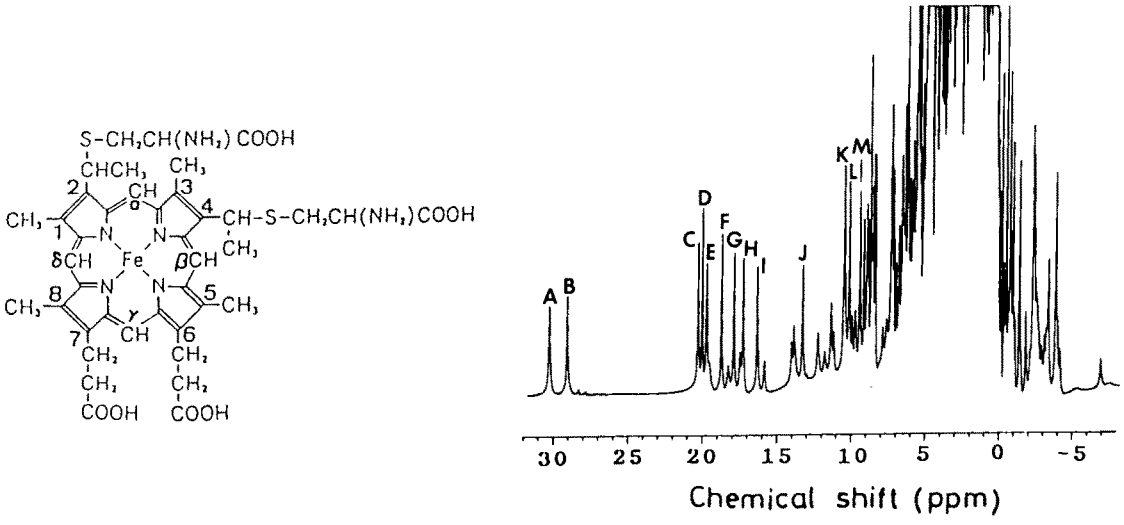

Fig. 1. A 400 MHz ^1H NMR spectrum of ferricytochrome c_3 from *D.v.* MF at 30°C. The heme methyl signals are labeled alphabetically. The chemical structure of a *c*-type heme and the labels of the porphyrin carbons are given on the left.

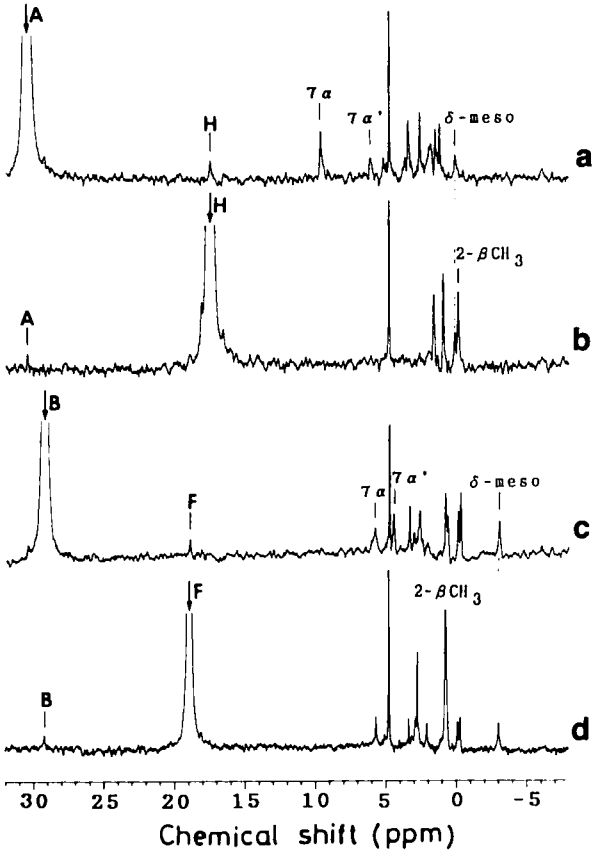

Fig. 2. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. A definition of the labels is given in Fig. 1. The label 7α denotes the α-methylene proton of the propionate at position 7. In the case of a, b and c, 17000 transients were accumulated.

They were classified into four hemes on the basis of the electron distribution probabilities in the five macroscopic oxidation states (Fan et al., 1990a), namely: heme 1 – A, H, I and K; heme 2 – B, F, G and M; heme 3 – C, D and J; and heme 4 – E and L, where the hemes are numbered according to the order in which their reduction takes place. La Mar and co-workers reported that the NOE between the methyl protons at positions 1 and 8 of a paramagnetic heme can be used for the assignment of methyl signals (Ramaprasad et al., 1984). It was applied to cytochrome c_3 from *D. desulfuricans* Norway by Guerlesquin et al. (1985a). We have carried out NOE experiments, irradiating heme methyl signals A through H one by one. As shown in Fig. 2, intraheme NOEs were observed for the pairs A and H, and B and F. Although the NOE signals were weak, they could be reproducibly observed. The former has already been reported by Fan et al. (1990a). Thus, signals A, B, F and H should belong to heme methyl groups at either position 1 or 8. Since NOE signals were observed at the α-methylene signals of the propionate group for signals A and B (Fig. 2), they can be ascribed to methyl groups at position 8 (8-CH_3), leading to the assignment of signals F and H to 1-CH_3. The assignment of the proprionate protons was confirmed by 2D

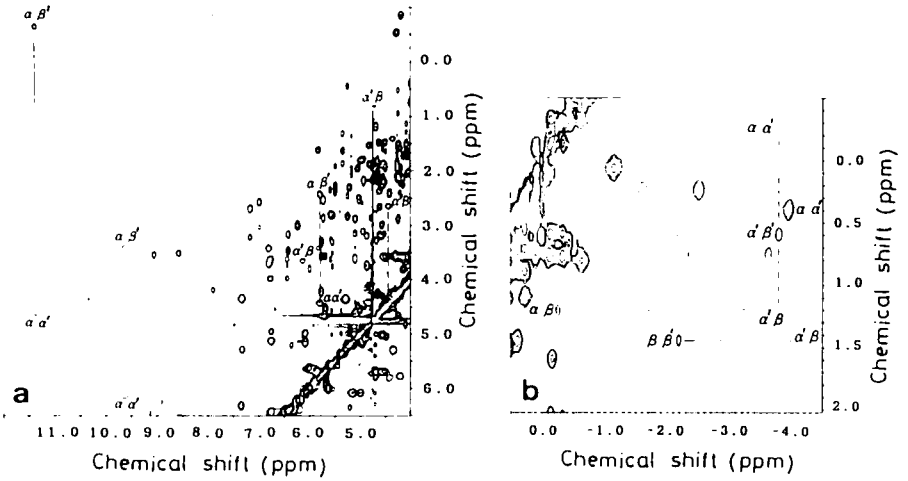

Fig. 3. Sections of the 2D TOCSY (HOHAHA) spectrum of ferricytochrome c_3 at 30°C. (a) the dotted and broken lines connect αCH-βCH cross peaks of 7-propionate of hemes 1 and 2 (related to signals A and B), respectively. Solid lines connect those of 6-propionate of heme 3 (related to signal C). (b) the broken and solid lines connect αCH-βCH cross peaks of 6-propionate of hemes 1 and 2 (related to signals I and G), respectively.

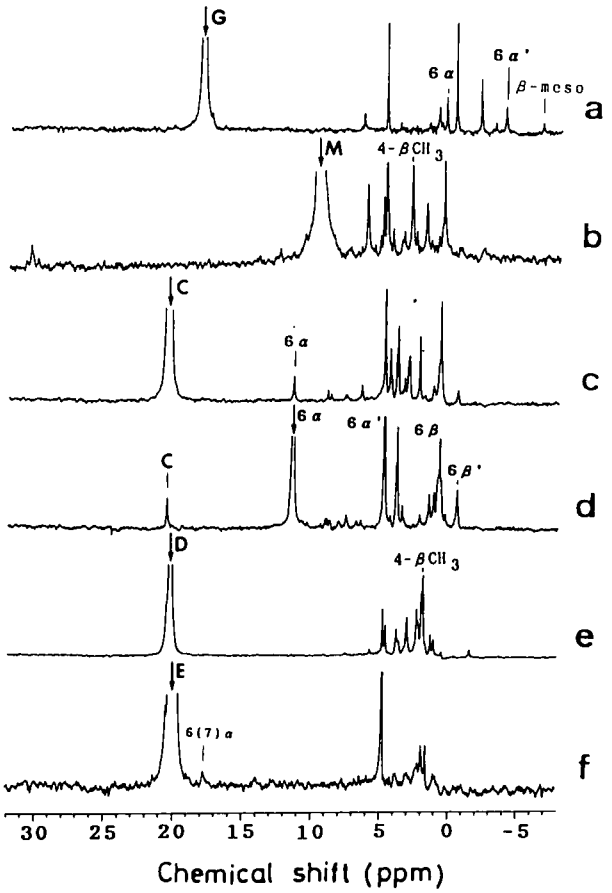

Fig. 4. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. A definition of the labels is given in Fig. 1. The labels 6α and 6β denote the α- and β-methylene protons of the propionate at position 6, respectively.

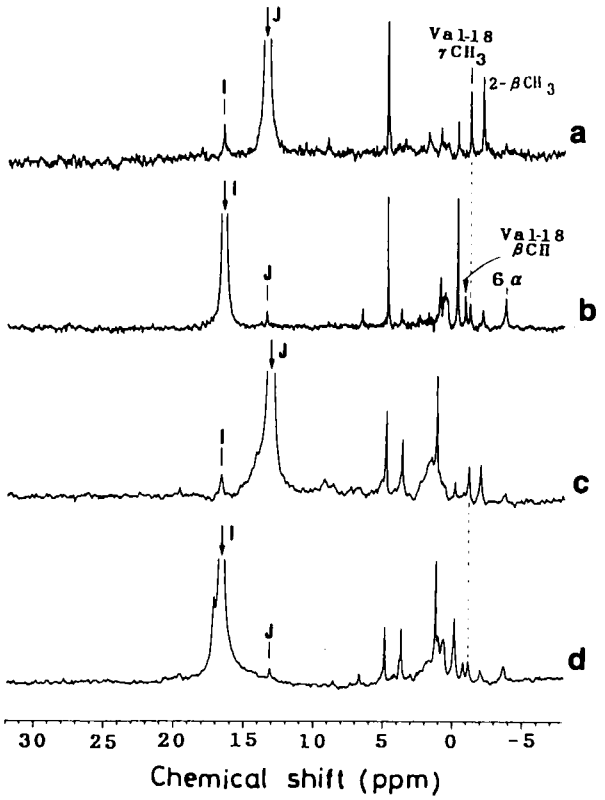

Fig. 5. NOE difference spectra of ferricytochrome c_3. The arrows indicate the irradiated positions. A definition of the labels is given in Fig. 1. (a) and (b), in aqueous solution; (c) and (d), in the presence of 50% (*v*/*v*) of deuterated ethanol.

DQFCOSY and TOCSY spectra. A relevant portion of the 2D TOCSY spectrum is presented in Fig. 3. The NOE signals observed in Figs. 2a and b, and in Figs. 2c and d were assigned to δ-mesoprotons of hemes 1 and 2, respectively. The irradiation of signals G, C, E and I also gave rise to NOE signals at the α-methylene protons of the propionate groups (Figs. 4 and 5), which were also confirmed by the 2D DQFCOSY and TOCSY spectra (Fig. 3). Thus, signals I and G can be attributed to 5-CH_3, leading to the assignment of K and M to 3-CH_3. Signals C and E should be due to either 5- of 8-CH_3. Irradiation of signals H, F, M, D and J gave rise to NOE signals at the β-methyl protons of the thioether bridges (Figs. 2, 4 and 5). The assignment of these signals was confirmed by the 2D DQFCOSY and TOCSY spectra through the connectivity with α-methine protons. These NOEs are consistent with the assignments of signals H, F and M mentioned above. Signal J can be assigned to 1-CH_3, as will be discussed later, leading to the assignment of D to 3-CH_3. Since irradiation of signal J did not induce an NOE at C, signal C should be attributed to 5-CH_3. The signal at −6.68 ppm in Fig. 4a was assigned to the β-mesoproton because irradiation of this signal gave rise to NOE signals at G and 4-αCH. On irradiation at 4-αCH, NOE signals were observed at the β-mesoproton and 4-βCH_3, which also confirmed the assignment of the 4-βCH_3 signal. These assignments are summarized in Table 1.

TABLE I
RESONANCE ASSIGNMENTS FOR HEME PROTONS IN *D.v.* MF FERRICYTOCHROME c_3 AT p^2H 7.0 AND 30°C

Heme number in NMR	Signals	Assignment	Chemical shift (ppm)		Heme number in crystal
1	A	8-CH_3	30.46		I
	H	1-CH_3	17.47		
	I	5-CH_3	16.51		
	K	3-CH_3	10.64		
		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		
2	B	8-CH_3	29.27		
	F	1-CH_3	18.92		
	G	5-CH_3	18.07		
	M	3-CH_3	9.60		
		β-meso	−6.68		
		δ-meso	−3.05		
		6-αCH_2	0.41	−3.92	
		6-βCH_2		−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		
3	C	5-CH_3	20.49		IV
	D	3-CH_3	20.21		
	J	1-CH_3	13.46		
		6-αCH_2	11.36	4.67	
		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		
4	E	5 or 8-CH_3	19.91		
	L	1 or 3-CH_3	10.30		
		6(or 7)-αCH_2	17.67	10.05	
		6(or 7)-βCH_2	0.08	−1.18	

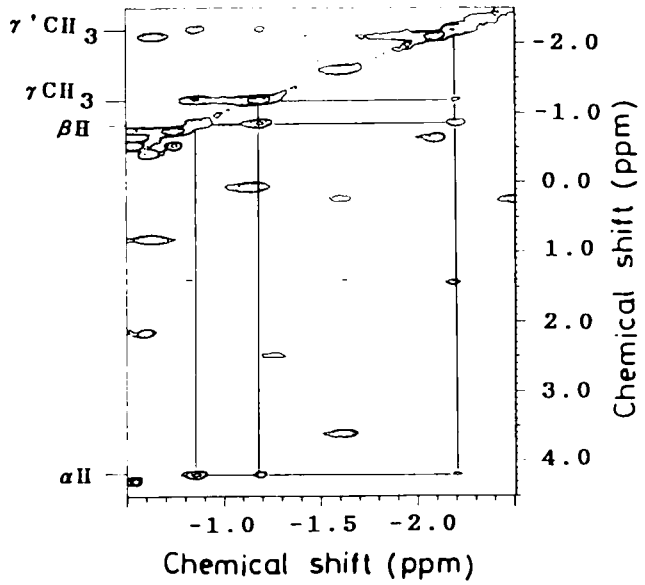

Fig. 6. A section of the 2D TOCSY (HOHAHA) spectrum of ferricytochrome c_3 at 30°C, showing the J-connectivity for the side-chain spin system of a valine residue, which was assigned as Val[18] (see text).

Assignment of heme groups

Gayda et al. (1987) assigned heme 3 to heme II (the Roman numeral is the numbering in the crystal structure) on the basis of an ESR study on *D.v.* MF cytochrome c_3. In contrast, Fan et al. (1990a) assigned heme 3 to heme IV on the basis of NMR measurements. To establish which assignment is correct, 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 0.417 nm, 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, signals I and J were assigned to 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively (Fan et al., 1990a). The interheme NOE between signals I and J was also confirmed in this work (Figs. 5a,b). The conformation in aqueous solution, however, may be different from that in the crystalline state, because the crystal was obtained by adding ethanol to an aqueous solution (Higuchi et al., 1984). Therefore, the effect of ethanol was examined. The NOE between signals I and J did not disappear, even in the presence of 50% (v/v) ethanol (Figs. 5c,d). Most cytochrome c_3 precipitated at 60–65% ethanol. Thus, it is unlikely that the conformation in the crystalline state is significantly different from that in aqueous solution, as far as the intermethyl distance of interest is concerned.

New and independent proof for the assignments of signals I and J was the NOE between the heme methyl groups and a neighboring amino-acid residue. The NOE signals at −1.21 and −0.85 ppm in Figs. 5a, b, c and d could be assigned to the γ-methyl and β-methine protons of a valine residue on the basis of the connectivity in 2D DQFCOSY and TOCSY spectra (Fig. 6). Val[18] is the only valine residue, which is located in the vicinity of the two different heme groups

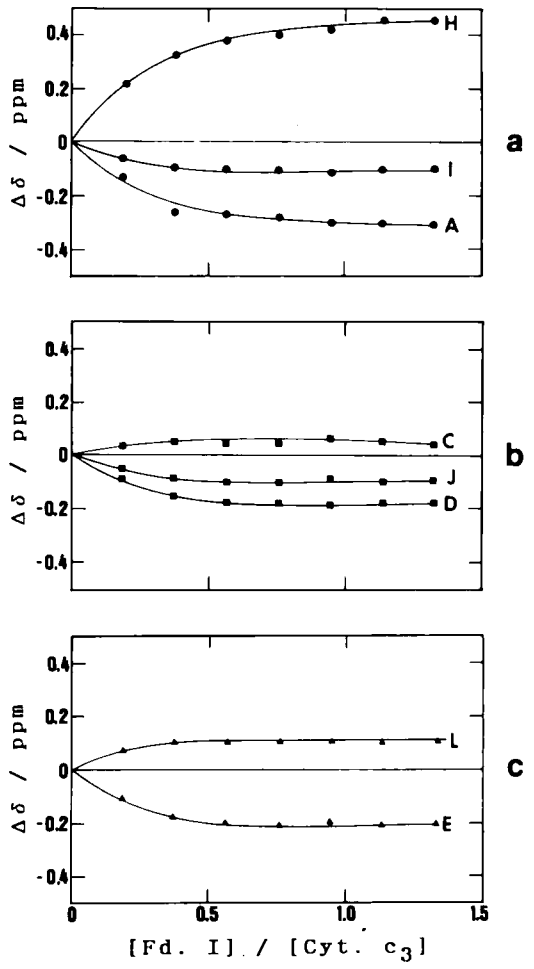

Fig. 7. The chemical shift changes of heme methyl resonances of ferricytochrome c_3 as a function of ferredoxin I (monomer) per cytochrome c_3. (a), (b) and (c), resonances belonging to hemes 1 (I), 3 (IV) and 4, respectively. Roman numerals in parentheses are the numbering in the crystal structure.

in the crystal structure. The carbon of γ-CH_3 of Val^{18} is located at distances of 0.432 and 0.371 nm from 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively. Furthermore, the intercarbon distance between 5-CH_3 of heme I and the β-methine of Val^{18} is 0.399 nm. These distances are completely consistent with the observed NOE network. Therefore, the NOE signals can be ascribed to Val^{18}, and signals I and J were confirmed to be due to 5-CH_3 of heme I and 1-CH_3 of heme IV, respectively. The third piece of evidence supporting the assignments of signals I and J is that they were actually assigned to 5-CH_3 and 1- or 3-CH_3 of different heme groups, respectively, on the basis of intraheme NOEs, as mentioned in the previous section.

In conclusion, signals I and J can be definitely assigned to the 5-methyl protons of heme I and 1-methyl protons of heme IV, respectively, which leads to the assignment of hemes 1 and 3 to hemes I and IV, respectively.

Interaction with ferredoxin I

^{1}H NMR spectra of cytochrome c_3 mixed with ferredoxin I at a variety of molar ratios were obtained. 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 in Fig. 7 as a function of the molar ratio of ferredoxin I to cytochrome c_3. The largest and second-largest changes were observed for signals H and A, respectively. Otherwise, the changes were smaller than 0.2 ppm. Since the signals of heme 2 changed little, they are not included in Fig. 7.

DISCUSSION

The NOE network among heme methyl signals I and J, and valine signals were found to correspond well to the distance network in the crystal structure of cytochrome c_3. This was also the case in the presence of 50% ethanol. These facts strongly suggest that the conformations of the core part of the protein in aqueous solution and the crystalline state are similar to each other. This justifies the elucidation of NOEs with reference to the crystal structure. The assignments of ^{1}H NMR signals in this work fully supported the site-specific heme assignments by Fan et al. (1990a).

The heme assignment mentioned above can be applied to analysis of the interaction of cytochrome c_3 with ferredoxin I. It is interesting to see the mechanism of interaction between multi-redox-site proteins. On the addition of ferredoxin I, the largest chemical shift changes were observed for signals H and A of cytochrome c_3. Both of them belong to heme I and are exposed to the solvent. This strongly suggests that the interaction site of cytochrome c_3 is heme I. The data for signal H in Fig. 7 were analyzed by nonlinear-least-square fitting on the basis of a 1:1 complex model, which did not work. However, a simulation based on the stoichiometry of two cytochrome c_3 molecules per monomer of ferredoxin I gave a good fit, as shown in Fig. 8. The association constant was estimated to be 10^8 M^{-2} at an ionic strength of 53 mM and 25°C. Although only one redox site of cytochrome c_3 is mainly involved in the binding, all the redox sites are used in the case of ferredoxin I.

Guerlesquin et al. investigated the interaction between cytochrome c_3 and ferredoxin I from *D. desulfuricans* by means of NMR (1985b) and calorimetry (1987). The stoichiometry of the complex

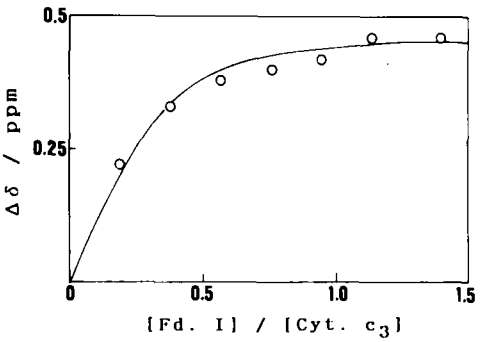

Fig. 8. Simulation of the titration curve for signal H of ferricytochrome c_3. The solid line represents the simulation curve with the stoichiometry of two cytochrome c_3 molecules per monomer of ferredoxin I and an association constant of 10^8 M^{-2}.

was one molecule of cytochrome c_3 per monomer of ferredoxin I. Since ferredoxin I from *D. desulfuricans* contains only one Fe-S cluster, the stoichiometry is four hemes per Fe-S cluster. Ferredoxin I from *D.v.* MF contains two Fe-S clusters (Okawara et al., 1988). Therefore, although the molecular stoichiometry in the case of *D.v.* MF was different from that in the case of *D. desulfuricans*, its redox-site stoichiometry was also four hemes per Fe-S cluster. The redox potentials of the two Fe-S clusters of ferredoxin I were reported to be higher and lower than that of heme I (Ogata et al., 1988). The two redox-sites could be the pathway for the forward and backward electron flow in the in vitro electron exchange. Guerlesquin et al. (1985b, 1987) obtained an association constant of 10^4 M^{-1} by NMR and one of 10^6 M^{-1} by calorimetry. They attributed the origin of the difference in the association constant to the difference in protein concentration (Guerlesquin et al., 1987). The former is in good agreement with ours, provided that the two binding sites of *D.v.* MF ferredoxin I have the same association constant. Judging from the chemical shift changes, the major interaction site of cytochrome c_3 is heme I for both binding sites. In the case of *D. desulfuricans*, the chemical shifts of hemes with the highest and second-highest redox potentials changed to similar extents on the complex formation in spite of the 1-to-1 stoichiometry (Guerlesquin et al., 1985b). In the crystal structure of cytochrome c_3 from *D.v.* MF, heme I is surrounded by the highest density of positive charges. Since ferredoxin I is an acidic protein, electrostatic interactions would be involved in the binding. Actually, weakening of the binding was observed with an increase in ionic strength (data not shown). For the interaction between cytochrome c_3 and ferredoxin from *D. desulfuricans*, it was speculated that heme 4 (corresponding to heme I in the *D.v.* MF cytochrome c_3 crystal) is also the most favorable interaction site (Cambillau et al., 1988). Guerlesquin et al. (1987), however, indicated that the interaction is essentially hydrophobic, in spite of the involvement of electrostatic interactions, because it is an entropy-driven reaction. The interaction of cytochrome c_3 with acidic proteins such as flavodoxin and rubredoxin has also been investigated experimentally (Stewart et al., 1989a) and by means of computer graphics (Stewart et al., 1989b). These proteins are also anticipated to interact with heme I.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, and Special Coordination Funds of the Science and Technology Agency of Japan. The authors are also grateful to Bruker Japan Co., Ltd. for their assistance.

REFERENCES

Benosman, H., Asso, M., Bertrand, P., Yagi, T. and Gayda, J.P. (1989) *Eur. J. Biochem.*, **182**, 51–55.
Cambillau, C., Frey, M., Mosse, J., Guerlesquin, F. and Bruschi, M. (1988) *Protein Struct. Funct. Genet.*, **4**, 63–70.
Fan, K., Akutsu, H., Kyogoku, Y. and Niki, K. (1990a) *Biochemistry*, **29**, 2257–2263.
Fan, K., Akutsu, H., Niki, K., Higuchi, N. and Kyogoku, Y. (1990b) *J. Electroanal. Chem.*, **278**, 295–306.
Gayda, J.P., Yagi, T., Benosman, H. and Bertrand, P. (1987) *FEBS Lett.*, **217**, 57–61.
Guerlesquin, F., Bruschi, M. and Wüthrich, K. (1985a) *Biochim. Biophys. Acta*, **830**, 296–303.
Guerlesquin, F., Noailly, M. and Bruschi, M. (1985b) *Biochem. Biophys. Res. Commun.*, **130**, 1102–1108.
Guerlesquin, F., Sari, J-C. and Bruschi, M. (1987) *Biochemistry*, **26**, 7438–7443.
Higuchi, Y., Kusunoki, M., Matsuura, Y., Yasuoka, N. and Kakudo, M. (1984) *J. Mol. Biol.*, **172**, 109–139.
Niki, K., Kawasaki, Y., Higuchi, Y. and Yasuoka, N. (1987) *Langmuir*, **3**, 982–986.
Ogata, M., Kondo, S., Okawara, N. and Yagi, T. (1988) *J. Biochem.*, **103**, 121–125.

Okawara, N., Ogata, M., Yagi, T., Wakabayashi, S. and Matsubara, H. (1988) *J. Biochem.*, **104**, 196–199.
Pettigrew, G.W. and Moore, G.R. (1987) *Cytochromes c: Biological Aspects*, Springer, Berlin, p. 19.
Pierrot, M., Haser, R., Frey, M., Payan, F. and Astier, J.P. (1982) *J. Biol. Chem.*, **257**, 14341–14348.
Postgate, J.R. (1984) *The Sulphate-reducing Bacteria*, 2nd ed., Cambridge University Press, Cambridge, U.K., p. 76.
Ramaprasad, S., Johnson, R.D. and La Mar, G.N. (1984) *J. Am. Chem. Soc.*, **106**, 3632–3635.
Santos, H., Moura, J.J.G., LeGall, J. and Xavier, A.V. (1984) *Eur. J. Biochem.*, **141**, 283–296.
Shinkai, W., Hase, T., Yagi, T. and Matsubara, H. (1980) *J. Biochem.*, **87**, 1747–1756.
Stewart, D.E., LeGall, J., Moura, I., Moura, J.J.G., Peck, H.D., Jr., Xavier, A.V., Weiner, P.K. and Wampler, J.E. (1989a) *Eur. J. Biochem.*, **185**, 695–700.
Stewart, D.E., LeGall, J., Moura, I., Moura, J.J.G., Peck, H.D., Jr., Xavier, A.V., Weiner, P.K. and Wampler, J.E. (1989b) *Biochemistry*, **27**, 2444–2450.
Yagi, T. and Maruyama, K. (1971) *Biochim. Biophys. Acta*, **243**, 214–224.