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PROPERTIES OF AROMATIC RESIDUES IN FERRICYTOCHROME c_3 OF *DESULFOVIBRIO VULGARIS* MIYAZAKI F STUDIED BY ^{1}H NMR*

JANG-SU PARK, MINORU ENOKI, AYAKO OHBU, KEJUNG FAN, KATSUMI NIKI and HIDEO AKUTSU**

Department of Physical Chemistry, Faculty of Engineering, Yokohama National University, Hodogaya-ku, Yokohama 240 (Japan)

YOSHIMASA KYOGOKU

Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)

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ABSTRACT

Conditions for the specific labelling of the tetrahaeme protein cytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F during culture of this sulphate-reducing bacterium in a minimal medium were established. Phenylalanine and tyrosine residues were specifically deuterated at more than 85% efficiency. Cytochrome c_3 has nine histidine, three tyrosine and two phenylalanine residues. Eight histidine imidazoles are ligated to four haeme groups. Using the deuterated cytochrome c_3, aromatic proton signals of phenylalanine and tyrosine residues in the fully oxidized state were identified. However, the signals of one phenylalanine residue were missing and this was tentatively assigned to Phe20. The aromatic proton signals of His67 were also assigned by p^{2}H titration. Its pk_a was much higher than that for the free histidine residue. No tyrosine residue was ionized up to p^{2}H 12.

INTRODUCTION

Cytochrome c_3 of *Desulfovibrio vulgaris* Miyazaki F is a tetrahaeme protein of molecular weight 14 000. It is involved in the electron-transport system of sulphate-reducing bacteria as a partner of hydrogenase enzymes. Although extensive investigations of this protein have been carried out by many different physicochemical methods, the significance of the four haemes is not yet elucidated. Its four macroscopic and 32 microscopic redox potentials were estimated from electrochemical and NMR results [1–3]. The crystal structure was reported at 1.8 Å resolution by Higuchi et al. [4]. The crystal data permit investigation of the relationship between its structure and redox behaviour.

*Dedicated to Professor Masamichi Tsuboi on the occasion of his 65th birthday.

**Author for correspondence.

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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 haemes are imidazole rings of histidine residues. The cytochrome c_3 molecule carries two phenylalanine, three tyrosine and one non-ligated histidine residues, which are located in the vicinity of haeme cavities. Tyr65 (tyrosine at the 65th position of the amino acid sequence) and Tyr66 are found in the vicinity of haeme I, and Phe76 and His67 are close to haeme II. Phe20 is between haemes III and IV and is conserved in all the sequences of cytochrome c_3 examined so far. Tyr43 is located in the vicinity of haeme IV. Some of these aromatic rings may be involved in the inter- and intra-molecular electron transfer.

If proton NMR signals of these aromatic rings are assigned, they can serve as good probes to monitor the behaviour of these groups in electron-transfer reactions. However, the assignment is not easy, because many signals overlap in the aromatic region and aromatic signals are scattered due to the paramagnetic effects of iron atoms and the ring current of the porphyrin nucleus. We have assigned the phenylalanine and tyrosine signals following specific deuteration of these residues in cytochrome c_3. Since the conditions for culture in minimal medium were not known, these were determined first. This was done successfully and all the aromatic proton signals in the spectrum of ferricytochrome c_3 were identified.

MATERIALS AND METHODS

Deuteration of phenylalanine and tyrosine

The deuteration of all the aromatic protons of phenylalanine and the 3.5 protons of tyrosine was carried out according to published methods [5] with only minor modifications. In order to remove exchangeable protons, 10 g of phenylalanine were dissolved in a mixture of 30 ml of ^2H_2O (^2H; 99.8%, Showadenko) and 10 ml of 6 N ^2HCl (^2H; 99%, CIL). The solvent was removed on a rotary evaporator and the sample was dried further under vacuum. It was then dissolved in 80 g of 85% (w/w) $^2H_2SO_4$ (originally 97%, ^2H; 99%, CIL). The solution was incubated at 50°C for 72 h, diluted to 1 l with distilled water and sulphate ions removed by adding $Ba(OH)_2$. Deuterated phenylalanine was purified by recrystallization and the deuteration was repeated twice to give a total yield of deuterated phenylalanine of 87%. Judged from the 400 MHz NMR spectrum, 98% of the aromatic protons had been deuterated.

Tyrosine was deuterated as follows. To remove exchangeable protons, 15 g of tyrosine were dissolved in a mixture of 45 ml of ^2H_2O and 18 ml of 6 N ^2HCl and processed as described above. The dried tyrosine was dissolved in a mixture of ^2H_2O (19.5 ml) and original $^2H_2SO_4$ (4.5 ml) and the solution refluxed, with stirring under nitrogen flow, at 110°C for 20 h. After evaporation, 19.5 ml of ^2H_2O were added and the reaction was repeated. The deuterated tyrosine

was recovered by recrystallization at pH 5.5 in 92% yield. Judged from the 400 MHz NMR spectrum, 97% of the 3,5 protons of the aromatic group were deuterated.

Desulfovibrio vulgaris Miyazaki F was cultured in the C medium proposed by Postgate [6], unless otherwise mentioned. Cytochrome c_3 was purified by the method of Yagi et al. [7] modified to include purification on Mono S and Superose columns (Pharmacia) as the last steps. The purity index (A_{552}(red)/A_{280}(ox)) of the final sample was over 2.9. The purity was also confirmed by SDS–polyacrylamide gel electrophoresis.

NMR measurements

^{1}H NMR spectra at 500 and 400 MHz were measured with JEOL GX500 and Bruker AM400 NMR spectrometers, respectively. Cytochrome c_3 was dissolved in a 30 mM phosphate buffer (p^{2}H 7.0) solution. A 500 MHz two-dimensional COSY spectrum was measured with 512×2048 points and 25000 Hz spectral width at a protein concentration of 4 mM. Chemical shifts were shown relative to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) as internal standard. The value of p^{2}H was measured on a pH meter and cited without any correction. The ^{2}H NMR spectra at 55.3 MHz were obtained with a Bruker WM 360 wb NMR spectrometer.

RESULTS

A minimal medium for the growth of sulphate-reducing bacteria

It was found that *D. vulgaris* Miyazaki F (*D.v.*MF) can be cultured in a minimal medium as shown in Table 1. The composition of this medium was decided by reference to media C and G proposed by Postgate and Pfennig et al., respectively [6], and a minimal medium for *E. coli* [5]. A better yield was obtained in the presence of 0.05 g l^{-1} of yeast extract. The yield was about 70% of that obtained in medium C and this minimal medium was used for the specific deuteration of cytochrome c_3. For deuteration, 0.2 and 0.8 g l^{-1} of deuterated phenylalanine and tyrosine, respectively, were used in place of the non-deuterated analogues.

^{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 5–11 ppm, measured at 25 °C, is presented in Fig. 1 together with the spectrum of the non-deuterated analogue. Signals at 7.6, 8.8 and 9.5 ppm were not observed for the deuterated compound, so these can be assigned to aromatic protons of phenylalanine res-

TABLE 1

A minimal medium (adjusted to pH 7.5) for *D. vulgaris* Miyazaki F (per liter)[a]

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	4.5 g
$CaCl_2 \cdot 6H_2O$	0.06 g
$MgSO_4 \cdot 7H_2O$	0.06 g
Sodium lactate	6.0 g
$FeSO_4 \cdot 7H_2O$	4.0 mg
Sodium citrate$\cdot 2H_2O$	0.3 g
Selenite solution[b]*	1.0 ml
Trace elements[c]*	1.0 ml
Vitamins[d]*	0.1 ml
Growth stimulants[e]*	0.1 ml
Other acids[f]*	0.1 ml
Amino acid mixture	
Ala	0.2 g
Arg	0.2 g
Asn	0.04 g
Asp	0.4 g
Cys	25 mg
Gln	0.1 g
Glu	1.0 g
Gly	0.1 g
Ile	0.3 g
Leu	0.4 g
Lys	0.45 g
Met	0.1 g
Phe	0.2 g
Pro	0.4 g
Ser	0.3 g
Thr	0.2 g
Val	0.3 g
His	0.15 g
Tyr	0.4 g
Trp	0.1 g

[a]Sterilized by autoclaving; components marked * added aseptically later. [b]From autoclaved stock of Na_2SeO_3 3 mg + NaOH 0.5 g l^{-1}. [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; $NiCl_2 \cdot 6H_2O$, 25 mg; $CuCl_2 \cdot 2H_2O$, 15 mg; $NaMoO_4 \cdot 2H_2O$, 25 mg l^{-1}. [d]From filter-sterilized stock of biotin, 1 mg; *p*-aminobenzoic acid, 5 mg; vitamin B_{12}, 5 mg; thiamine, 10 mg per 100 ml. [e]From autoclaved stock of isobutyric acid, valeric acid, 2-methylbutyric acid, 3-methylbutyric acid, 0.5 g each; caproic acid, 0.2 g; succinic acid, 0.6 g per 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 per 100 ml, NaOH to pH 9.

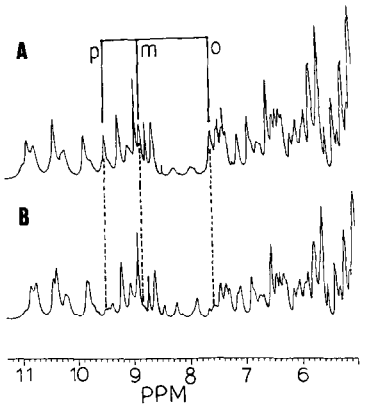

Fig. 1. 500 MHz ^{1}H NMR spectra of ferricytochrome c_3 at 25 °C. A, normal; B, specifically deuterated on phenylalanine residues. The assignment of the *ortho*, *meta* and *para* ring protons of phenylalanine is given as *o*, *m* and *p*, respectively, in the figure.

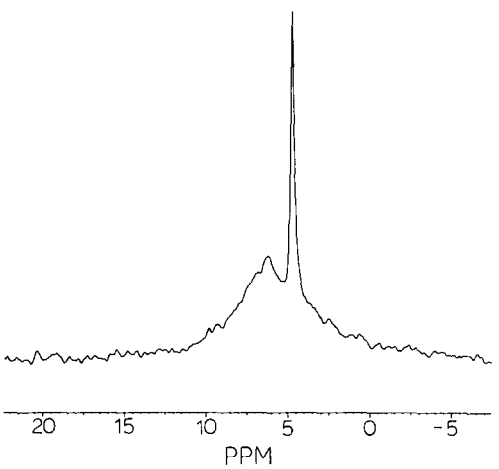

Fig. 2. 55.3 MHz ^{2}H NMR spectrum of ferricytochrome c_3 specifically deuterated on phenylalanine residues at 30 °C. The sharp signal is due to water.

idues. From the integrated intensity, the extent of deuteration was estimated to be about 85%.

*D.v.*MF cytochrome c_3 has two phenylalanine residues, namely, Phe20 and Phe76. They should give rise to more than six signals. The results in Fig. 1 show that the signals of only one phenylalanine residue 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 anywhere in the spectrum. A ^{2}H NMR spectrum of DPhe-cyt c_3 at 55.3 MHz is shown in Fig. 2. A broad signal with a peak

at 6.5 ppm was observed apart from the water signal. The weight-averaged chemical shift of the three phenylalanine signals in Fig. 1 was about 8.5 ppm. This suggests that the signals due to the other phenylalanine residue should be located at higher field. The assigned phenylalanine signals were also observed in the two-dimensional COSY spectrum at 50 °C shown in Fig. 3. It is clear from this spectrum that the central signal (8.8 ppm at 25 °C) is coupled with the other two. Therefore, the signals at 7.6 and 8.8 ppm can be assigned to 2,6(or *ortho*) and 3,5(or *meta*) ring protons, respectively. This was also confirmed by the one-dimensional 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 broadening of its 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.

A 400 MHz ^{1}H NMR spectrum of ferricytochrome c_3 specifically deuterated at tyrosine residues (DTyr-cyt c_3) in the region from 5 to 11 ppm is presented in Fig. 4, together with a spectrum of the non-deuterated analogue and the difference spectrum. These were measured at 50 °C to provide better resolution. The difference spectrum clearly shows that three signals at 5.7, 6.3 and 6.6 ppm are absent from the deuterated spectrum. These were assigned to the

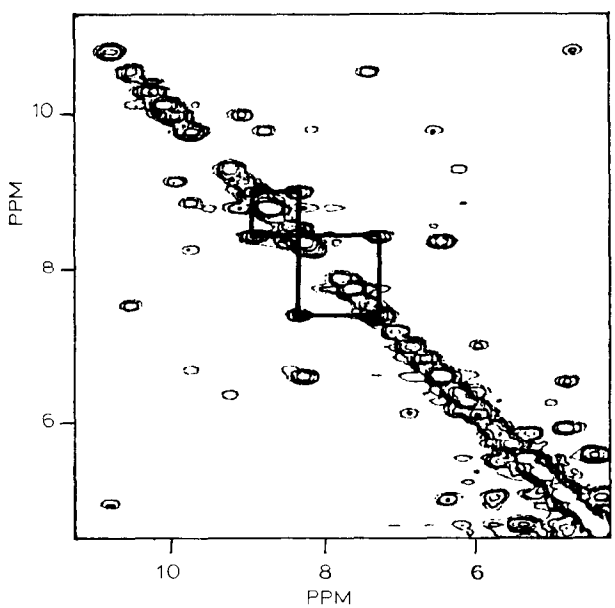

Fig. 3. 500 MHz two-dimensional COSY spectrum of ferricytochrome c_3 in the aromatic region at 50 °C. The connectivity of phenylalanine signals is shown by solid lines.

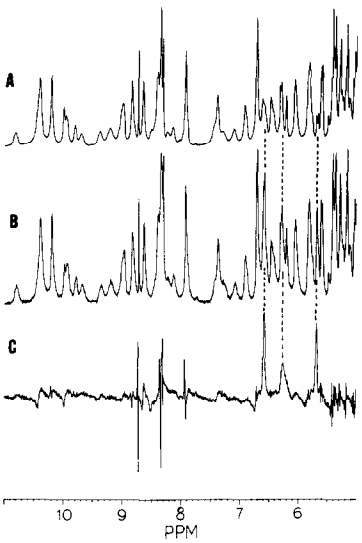

Fig. 4. 400 MHz ^{1}H NMR spectra of ferricytochrome c_3 specifically deuterated on tyrosine residues (A); non-deuterated (B), and their difference spectrum (C) at 50 °C.

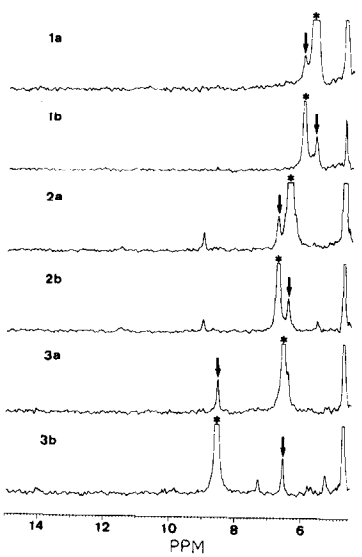

Fig. 5. NOE difference spectra for identifying three sets of tyrosine ring signals of ferricytochrome c_3 at 30 °C. The asterisk and arrow indicate the irradiated position and relevant NOE signal, respectively. Three pairs of signals from tyrosine residues were identified by the experiments 1(a, b), 2(a, b) and 3(a, b), respectively. Sixteen free-induction decays (FID) were accumulated, respectively under on-resonance and off-resonance irradiation for 0.5 s. In total, 4800 transients were accumulated for each FID.

3,5 ring protons of tyrosine residues, because only those positions were deuterated. The efficiency of deuteration was much better than for phenylalanine. Since *D.v.*MF cytochrome c_3 has three tyrosine residues, all of them were identified in the spectrum. The signals from the 2,6 ring protons of tyrosine were identified by NOE by irradiation of the 3,5 protons and confirmed by irradiation of possible 2,6 ring protons. Relevant NOE difference spectra at 30 °C are summarized in Fig. 5.

Three pairs of signals from tyrosine residues were identified at (6.5, 8.5), (6.4, 6.7) and (5.6, 6.0) ppm for (3,5H, 2,6H) resonances at 30 °C. Since only two sets of signals were observed, it can be concluded that the flip rate of the aromatic ring is faster than the NMR time scale for all three tyrosine residues. The difference spectrum in Fig. 4 shows, however, that the linewidth of the signal at 6.3 ppm is much broader than those of the other signals, suggesting that the mobility of one tyrosine ring is suppressed compared with the others.

p^2H titration of phenylalanine and tyrosine signals

The ring protons of phenylalanine and tyrosine were titrated from p ^{2}H 4.5–12. Their chemical shifts were plotted as a function of p^{2}H in Fig. 6. No tyrosine

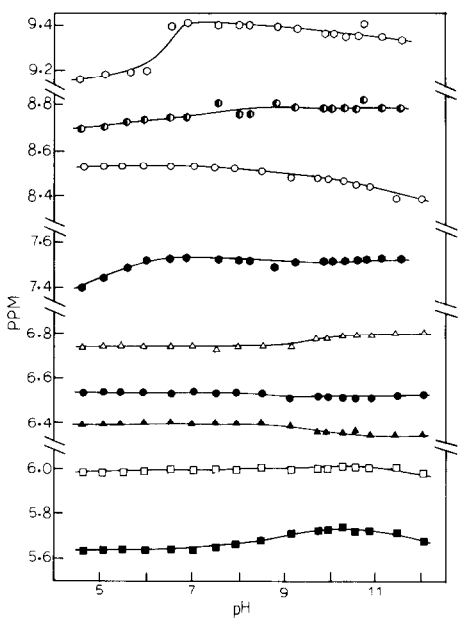

Fig. 6. p^{2}H dependence of the chemical shifts of phenylalanine and tyrosine ring protons of ferricytochrome c_3 at 30 °C. Open, half-closed and closed hexagons represent *para, meta* and *ortho* ring protons of phenylalanine, respectively. The other three pairs of symbols represent the three tyrosines. Open and closed symbols correspond to 2,6-H and 3,5-H resonances of tyrosine, respectively. The p^{2}H was adjusted by addition of a concentrated ^{2}HCl or NaO^{2}H solution.

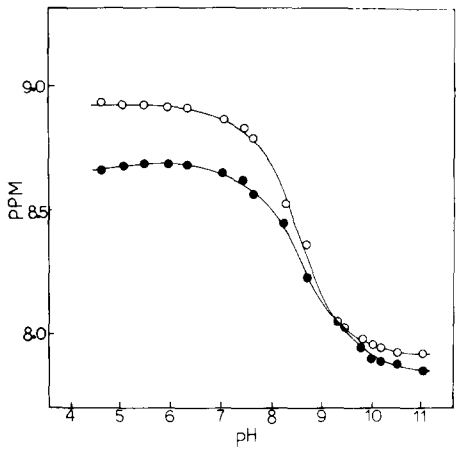

Fig. 7. p^2H dependence of chemical shifts of His67 ring protons at 30 °C. Open and closed circles represent C2-H and C4-H resonances of the imidazole ring.

residue was deprotonated in the region examined. The p^2H dependence was minor for all tyrosine ring protons, with only slight changes being observed in the alkaline region. In contrast, a rather large change can be seen in the acidic region for the 4-H of phenylalanine.

Assignment of nonligated histidine signals

The p^2H titration curves for sharp signals with single proton intensity in the aromatic region are presented in Fig. 7. Both protons show similar pK_a values and are assigned to a histidine residue. Actually, the intensity of the signal at lower field was found to decrease at p^2H 10–12. This proton was assigned to C2-H of the imidazole ring. The signal in the acidic region was assigned using cytochrome c_3 deuterated at the C2 position. Since cytochrome c_3 has only one non-ligated histidine residue, these signals were assigned to C2-H and C4-H of His 67. The pK_a values obtained by non-linear least-squares fitting were 8.51 and 8.67 for C2-H and C4-H, respectively. This pK_a is much higher than that of a free histidine residue [8] and the change in the chemical shift of C4-H during titration was also larger than normal.

DISCUSSION

Growth conditions for *Desulfovibrio vulgaris* Miyazaki F in a minimal medium were established in this work. The yield of cells and the efficiency of specific deuteration of cytochrome c_3 were satisfactory, at least for phenylalanine and tyrosine. This system can be used not only for the deuteration of

other amino acid residues but also for the labelling of proteins with other stable isotopes.

*D.v.*MF cytochrome c_3 has two phenylalanine residues, Phe20 and Phe76. Phe20 is the only residue which is conserved in all amino acid sequences of cytochrome c_3 determined so far. According to the crystal structure, it is between haemes III and IV, with its aromatic ring parallel to the porphyrin ring of haeme III and to the imidazole ring of His25, a ligand of haeme IV [4]. In contrast, Phe76 is located close to the surface of the protein so its ring is expected to be more mobile than that of Phe20. This leads to a tentative assignment of the observed signals to Phe76, an assignment which is supported by the titration curves of these signals which show a change in their chemical shifts in the acidic region. Actually, the ring of Phe76 is close to the propionates of haeme II in the crystal structure. Particularly, 4-H of the ring is very close to one of the carboxyl oxygens of the propionate at the 6 position. In contrast, there is no carboxyl group in the neighbourhood of the ring of Phe20. The broadening of the aromatic ring signals of a phenylalanine residue has also been reported for horse ferrocytochrome *c* [9]. Ring protons at *ortho* and *meta* positions of Phe82 could not be observed at 25°C, but did appear at above 85°C. Our tentative assignment suggests that the stacking of the aromatic rings of His25, Phe20 and haeme III cannot stop the flip rotation of the Phe20 ring.

The imidazole signals of His67 could be assigned by p^2H titration. Their pK_a and chemical shifts, however, were different from normal. Inspection of the crystal structure shows that the imidazole ring forms hydrogen bonds with the carboxyl groups of Asp71 and the propionate of haeme II. The same propionate also forms a hydrogen bond with the amide of Lys75 and this must be the major reason why His67 shows an abnormal pK_a. It has been reported that most haeme methyl signals shifted at around p^2H 8.5 in the p^2H titration [10]. The change was ascribed to a dissociative group with pK_a about 8.5, close to the observed pK_a value of His67 which could thus be responsible for the change. Thus, the chain of hydrogen bonds mentioned above could contribute significantly to the conformation of the entire protein. This is reasonable because the hydrogen-bonding chain is connected with the polypeptide backbone through the amide of Lys75. The change in chemical shift of C4-H during titration was much larger than normal which also suggests that the titration of His67 induces a conformational change around it.

Since the phenol groups of the three tyrosine residues were not ionized up to p^2H 12, all of them are expected to be either buried in the protein or interacting with other residues. In the crystal structure, Tyr65 is completely buried, while Tyr43 and Tyr66 are partially buried. Furthermore, the phenol groups of Tyr43 and Tyr65 form hydrogen bonds with carboxyl groups. All this is in accordance with the results of p^2H titration. However, the OH group of Tyr66 is apparently exposed on the surface in the crystal structure. Although it is not

clear why this was not ionized, it might be that the group is involved in some interactions.

*D.v.*MF cytochrome c_3 contains 14 aromatic amino acid residues including eight ligated histidines. Thirteen out of 14 residues were found to be involved in some interactions. This would suggest that the aromatic residues play important roles in the structure formation and function of this protein. Our next target is to clarify their involvement in the electron transfer on the basis of the assignments established in this work.

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