Effects of charged amino-acid mutation on the solution structure of cytochrome *b*₅ and binding between cytochrome *b*₅ and cytochrome *c*

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Abstract

The solution structure of oxidized bovine microsomal cytochrome *b*₅ mutant (E48, E56/A, D60/A) has been determined through 1524 meaningful nuclear Overhauser effect constraints together with 190 pseudocontact shift constraints. The final family of 35 conformers has rmsd values with respect to the mean structure of 0.045±0.009 nm and 0.088±0.011 nm for backbone and heavy atoms, respectively. A characteristic of this mutant is that of having no significant changes in the whole folding and secondary structure compared with the X-ray and solution structures of wild-type cytochrome *b*₅. The binding of different surface mutants of cytochrome *b*₅ with cytochrome *c* shows that electrostatic interactions play an important role in maintaining the stability and specificity of the protein complex formed. The differences in association constants demonstrate the electrostatic contributions of cytochrome *b*₅ surface negatively charged residues, which were suggested to be involved in complex formation in the Northrup and Salemm models, have cumulative effect on the stability of cyt *c*-cyt *b*₅ complex, and the contribution of Glu48 is a little higher than that of Glu44. Moreover, our result suggests that the docking geometry proposed by Northrup, which is involved in the participation of Glu48, Glu56, Asp60, and heme propionate of cytochrome *b*₅, do occur in the association between cytochrome *b*₅ and cytochrome *c*.

Keywords: Cytochrome *b*₅; cytochrome *c*; mutant; NMR; solution structure; electrostatic interaction; binding

Supplemental material: See www.proteinscience.org.

Electron transfer reactions between metalloproteins play critical roles in numerous important biological processes such as photosynthesis, oxidative phosphorylation, and xenobiotic processing (Marcus and Sutin 1985; Dreyer 1984).

The interaction between cyt *b*₅ and cyt *c* is an excellent model system for investigating fundamental questions regarding interprotein electron transfer. The experimental and theoretical investigation of the interaction of two such proteins has provided a considerable insight into the initial characterization of the nature of this complex and the manner in which two proteins recognize and bind to each other (Mauk et al. 1995; Rodgers et al. 1988; Burch et al. 1990). However, the question is still open as to the surface region of cyt *b*₅ involved in the recognition of cyt *c* and the forces contributing significantly to the stability of the complex. The model obtained by the Brownian dynamic simulations predicted that the two proteins approach each other with two

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Abbreviations: cyt *b*₅, cytochrome *b*₅; cyt *c*, cytochrome *c*; mutant I (cytochrome *b*₅ E48, E56/A, D60/A); mutant II (cytochrome *b*₅ E44, E56/A, D60/A); mutant III (cytochrome *b*₅ E44, E48, E56/A, D60/A).

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different docking geometries (Northrup et al. 1993). The first one involves the interactions of Glu48-Arg13, Glu56-Lys87, Asp60-Lys86, and heme propionate-Tml72. The second one is identical with the Salemme model (Salemme 1976) including the following salt bridges as Glu44-Lys27, Glu48-Lys13, Glu60-Lys72, and heme propionate-Lys79 (cyt *b*₅ residues listed first), electrostatic interaction at the molecular interface stabilizes the association complexes. However, utilization of high-pressure techniques coupled with site-directed mutagenesis reached a conclusion that electrostatics did not provide the main stabilizing factors in the overall association of this protein-protein complex (Rodgers and Sligar 1991). Also, thermodynamic parameters such as Gibbs free energy and volume were nonadditive in relation to their corresponding single, double, or multiple-site charged residue's mutation. Recently, we had preliminary proof that electrostatic interaction might contribute considerably to macromolecular associations (Wu et al. 2001). To further investigate the electrostatic interactions on the cyt *c*-cyt *b*₅ complex formation, whether the electrostatic contributions of the surface-charged residues to the association of cyt *c* and cyt *b*₅ are cumulative, and whether the binding geometry proposed by Northrup et al. (1993), which differed from the Salemme model, exists in the solution, we employ a site-directed mutagenesis system to generate the following cyt *b*₅ mutants: mutant I (E48, E56/A and D60/A), mutant II (E44, E56/A and D60/A). Here, we report the high-quality, three-dimensional solution structure and magnetic susceptibility of mutant I, the binding between cyt *b*₅ mutants and cyt *c* characterized by high-resolution nuclear magnetic resonance (NMR). The comparison of association constants of mutant I-cyt *c* and mutant II-cyt *c* complex with those of mutant III (E44, E48, E56/A and D60/A)-cyt *c* and wild-type cyt *b*₅-cyt *c* complex previously reported further confirms that electrostatic interactions play an important role in maintaining the stability and specificity of the complex formed, the differences in association constants demonstrate the electrostatic contributions of the cyt *b*₅ surface negatively charged residues Glu44, Glu48, Glu56, and Asp60, which were thought to take participation in complex formation in the Northrup and Salemme models, are cumulative to the stability of cyt *c*-cyt *b*₅ complex. The binding geometry proposed by Northrup, which is involved in the following interactions (cyt *b*₅-cyt *c*) Glu48-Arg13, Glu56-Lys87, Asp60-Lys86, and heme propionate-Tml72 do occur in the solution of the wild-type system.

Results and Discussion

Sequence-specific assignment and secondary structure

In 10 mM phosphate buffer, pH=7.0, two forms exist in solution as a result of two conformations of the heme ring

differing by a 180° rotation around the α - γ axis of the heme, but only the major form is our aim.

In total, 77% of the expected proton resonances (except Ala3) were assigned (data shown in supplemental material). Figure 1 shows the short- and medium-range nuclear Overhauser effects (NOEs) observed for the backbone and β protons from NOESY maps in H₂O. Sequential d_{NN} connectivities were observed for residues Tyr7-Asn17, Lys19-His26, Val29-Glu38, Gly42-Gly52, Ala54-Gly62, Ser64-Ile76, Glu78-Leu79, and Asp82-Asp83. Missing connectivities could be because of the paramagnetism of the protein, the exchange region of some HN (amide hydrogen) groups, or the presence of prolines, which do not have an amide proton. The proton resonances of Ala3 were not determined from the spectra because of the fact that the amino-terminal residue is much more flexible and its amide proton exchanges rapidly with the solvent. The assignment of the resonances of the heme protons was performed mainly on the NOESY spectrum of spectral width of 42 ppm.

The elements of secondary structure can be identified by analyzing the pattern of assigned NOEs. In general, β strands are expected to give strong $d_{\alpha N}$ sequential and intraresidue connectivities and weak d_{NN} connectivities. The pattern of long-range NOEs (figure shown in supplemental

Fig. 1. Schematic representation of the sequential and medium-range nuclear Overhauser effect (NOE) connectivities involving NH, H α , and H β protons for the oxidized form of cyt *b*₅ mutant (E48, E56/A, D60/A). The thickness of the bar indicates the intensity of NOEs.

material) indicates the existence of a β -pleated region centered on two antiparallel β strands (residues 21–25 and 28–32), the former being parallel to segment 51–54, and the latter antiparallel to region 75–79. Finally, connectivities indicating an antiparallel β strand were observed for residues 78–80 and 5–7. Helical structures can be identified by the high number of sequential and medium-range connectivities such as $d_{NN}(i,i+1)$, $d_{NN}(i,i+2)$, $d_{\alpha N}(i,i+1)$, $d_{\alpha N}(i,i+3)$, $d_{\alpha N}(i,i+4)$, and $d_{\alpha\beta}(i,i+3)$. Six elements of helical secondary structure can be predicted similar to the characteristic secondary structural elements present in cyt *b₅* crystal structure (PDB accession number 1cyo) (Durley and Mathews 1996). They involve residues 9–15 ($\alpha 1$), 32–39 ($\alpha 2$), 43–49 ($\alpha 3$), 55–62 ($\alpha 4$), and 64–75 ($\alpha 5$).

Solution structure determination

A total number of 1973 experimental NOESY constraints were obtained, the major part taken from the mixing time of 100 ms NOESY in H_2O . Of these, 1524 constraints turned out to be meaningful (corresponding to 24 or 18.6 constraints per residue, respectively) and used in the structure calculations together with 190 pseudocontact shifts constraints. The number of experimental meaningful NOEs per residue is demonstrated in Figure 2A. A total of 34 stereospecific assignments were obtained through the program GLOMSA (data shown in supplemental material).

The 35 conformers obtained from PSEUDYANA with lowest target function constituted the final family. The family has rmsd values (hereafter, unless specified, rmsd values are calculated for residues 5–82) to the mean structure of 0.047 ± 0.008 nm for the backbone and 0.089 ± 0.010 nm for the heavy atoms. The average total target function value for the above 35 structures is 0.0025 ± 0.0005 nm², while the contribution of pseudocontact shift constraints to the target function is <10% with respect to that of NOE constraints. The 35 conformers were subjected to restrained energy minimization. The resulting family has rmsd values of 0.045 ± 0.009 nm and 0.088 ± 0.011 nm to the mean structure for backbone and all heavy atoms, respectively. The rmsd values per residue for the backbone and heavy atoms are reported in Figure 2B. The average penalty function of 43.88 kJ mol⁻¹ (31.56 – 55.50 kJ mol⁻¹) corresponds to a target function value of 0.0032 nm² (0.0024 – 0.0042 nm²). In addition, the contribution of pseudocontact shifts is lower than 5% compared to that of NOE constraints.

The quality of the structure in the terms of stereochemical parameters was checked with the programs PROCHECK (Laskowski et al. 1993) and PROCHECK-NMR (Laskowski et al. 1996). For the energy-minimized mean structure, the following residues are found to form α helices 9–14, 33–35, 43–49, 55–61, and 65–74. The short segment involving the C-terminus residues 81–83, which are part of the helix $\alpha 6$ (81–87) present in crystal, also shows helical

Fig. 2. The number of meaningful nuclear Overhauser effect (NOE)-derived constraints (A) is compared with the rmsd values (B) of the family. White, gray, dark gray, and black vertical bars represent respectively intra-residue, sequential, medium-range, and long-range connectivities. The data for His63 includes connectivities involving the heme moiety. ■ and ○ represent rmsd values per residue to the mean structure for the PSEUDO-REM family of structures for backbone and for all heavy atoms, respectively.

structure. Four segments of β secondary structures are identified for residues 5–7, 75–79, 28–31 and 20–25. Another β sheet involving 16–17, which exists in X-ray structure (PDB accession number 1cyo), also appeared while β sheet involving 51–54 was not found by the PROCHECK-NMR as reported before (Arnesano et al. 1998; Muskett et al. 1996). The differences may arise mainly from small changes in dihedral angles, which propagate along the structure and induce relative slight movement of the elements of secondary structures.

To give a clearer view about the structural similarity of our mutant and wild-type cyt *b₅*, the ribbon diagrams of the average energy-minimized structure of the mutant and the X-ray crystallographic structure of the wild type (PDB accession number 1cyo) are shown in Figure 3, together with a stereoview of the superimposed structures by superimpos-

Fig. 3. Ribbon diagrams of the average minimized solution structures of the bovine oxidized cyt b_5 mutant and X-ray structure of wild-type cyt b_5 (A and B, respectively). A stereoview of the superimposed structures of cyt b_5 mutant (light gray line) and the wild type (black line) is shown (C).

ing all heavy atoms (except the side chains of residues 48, 56, and 60) for residues 5–82 of these two structures. The global folds of the two structures still remain similar and most of the parts superimpose quite well. The rmsd between two structures is 0.074 nm and 0.131 nm for the backbone and heavy atoms, respectively, which clearly suggests that overall folding of cyt b_5 mutant (E48, E56/A, D60/A) was largely conserved in comparison with the structure of wild-type cyt b_5 , and mutations on the surface-charged residues have not perturbed the local conformation significantly. A comparison also was made between the mutant and the high-resolution solution structure of wild-type rat cyt b_5 (PDB accession number 1aw3) (Arnesano et al. 1998), which has a 94% homology with the wild-type bovine cyt b_5 . The rmsd between solution structures of rat cyt b_5 and the mutant is 0.085 nm for the backbone atoms, the overall fold of cyt b_5 is thus very well maintained in the different species.

Calculation statistics and the structure quality analysis for the solution structure are shown in Table 1. No distance violations are >0.3 , and no forbidden van der Waal's contacts are observed in both family and the average structure,

characterizing the good quality of the present solution structure. Figure 4 shows the structure of the family represented as a tube whose radius is proportional to the rmsd of the family. As to the backbone atoms, root mean square deviation (RMSD) fluctuations higher than the average were located to the following main regions: residues 16–20, region 1; 37–50, region 2; and 59–66, region 3. It is interesting to note the residues in these regions showed fast deuterium-exchange rates (Dangi et al. 1998), and molecular dynamics simulation also indicated these regions seemed to be dynamic (Storch and Daggett 1995). Residues in regions 2 and 3 are located mainly along the periphery of the heme pocket and contain most of the acidic residues that were thought to participate in interfacial interactions with cyt c in the Salemme and Northrup models. In regards to region 1, previous molecular dynamics simulation had implicated that the region might have a role in the free energy interaction with the partner protein and that it provided an alternative binding site for cyt b_5 (Hom et al. 2000).

Magnetic susceptibility tensor

The pseudocontact contribution arises from the magnetic susceptibility anisotropy and depends on the position of a nucleus with respect to the axes of the magnetic susceptibility tensor. Within the metal-centered point dipole-point dipole approximation, the following equation holds (Kurland and McGarvey 1970):

$$\delta_{pcs} = \frac{1}{12\pi r_i^3} \left[\Delta\chi_{ax}(3n_i^2 - 1) + \frac{3}{2} \chi_{rh}(l_i^2 - m_i^2) \right] \quad (1)$$

where $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic magnetic susceptibility anisotropies, r_i the length of the nuclei i from the metal ion, and l_i , m_i and n_i the direction cosines of the position vector of atom i with respect to the orthogonal reference system formed by the principal axes of the magnetic susceptibility tensor. By finding the best fit of equation 1 to a set of pseudocontact shifts, the tensor has $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ value of 2.88×10^{-32} and $-1.12 \times 10^{-32} \text{ m}^3$, respectively. The principal z -axis of the magnetic anisotropy tensor forms an angle $\sim 5.8^\circ$ perpendicular to the heme plane. The X -axis makes an angle of 21° with the α - γ axis. The values are in good agreement with those reported for solution structures of rat and rabbit cyt b_5 (Arnesano et al. 1998; Banci et al. 2000), showing mutation of three surface-charged residues had not perturbed the orientation of the ligands and electronic structure of the heme.

The orientation of the in-plane axes of the magnetic susceptibility tensor is known to be essentially dependent on the relative arrangement of the iron axial ligands (Shokhirev and Walker 1998), and in the present case, on the orientation of the imidazole planes of His39 and His63. The rota-

Table 1. Calculation statistics and structural analysis of solution structure of cyt *b*₅ mutant I

	Average over the family	Mean structure
RMS violations per experimental distance constraint (nm) ^a		
Intra-residue (299)	0.0017 ± 0.0002	0.0015
Sequential (384)	0.0013 ± 0.0003	0.0011
Medium range ^b (385)	0.0013 ± 0.0002	0.0012
Long range (456)	0.0009 ± 0.0002	0.0009
Total (1524)	0.0013 ± 0.0001	0.0012
Average number of violations per structure		
Intra-residue	8.43 ± 1.81	6
Sequential	7.86 ± 2.14	7
Medium range ^b	7.71 ± 2.00	9
Long range	6.28 ± 1.65	8
Total	30.3 ± 4.25	30
Violations larger than 0.3 Å	0.00 ± 0.00	0
Violations between 0.1–0.3 Å	7.42 ± 2.63	7
Target function (nm ¹)	0.0033 ± 0.0009	0.0026
AMBER force field average total energy (kJ mol ⁻¹)	-5281 ± 752	-5363.8
Structure precision (nm) ^c		
Backbone	0.045 ± 0.009	—
All heavy atoms	0.088 ± 0.011	—
Structure analysis ^d		
% of residues in most favored regions	83.3 ± 3.5	84.7
% of residues in additionally allowed regions	14.9 ± 3.9	13.9
% of residues in generously allowed regions	1.4 ± 1.5	1.4
% of residues in disallowed regions	0.8 ± 1.1	0.0
No. or bad contacts/100 residues	0.03	0.0
H-bond energy (kJ mol ⁻¹)	3.16	3.14
Overall G-factor	-0.15 ± 0.07	-0.11

^a The number of meaningful constraints for each class is reported in parentheses.

^b Medium-range distance constraints are those between residues (i, i + 2), (i, i + 3), (i, i + 4), and (i, i + 5).

^c rmsd values are calculated for residues 5–82.

^d The programs PROCHECK and PROCHECK-NMR were used to check the overall quality of the structure and Gly and Pro are excluded from the Ramachandran analysis. For the Procheck statistic, <10 bad contacts per 100 residues, an average hydrogen bond energy in the range of 2.5–4.0 kJ mol⁻¹ and an overall G-factor >–0.5 are expected for a good quality structure.

tion of the *Y*-axis of the tensor with respect to a given Fe-pyrrole I nitrogen direction is equal in magnitude, but opposite, to the rotation of the bisector of the normals to the two imidazole planes with respect to the same Fe-pyrrole I nitrogen direction (Banci et al. 2000). In all the 35 conformations of the family, the normal to the plane of His39 made an angle of 45° with the Fe-pyrrole I nitrogen direction, while the normal to the plane of His63 made an angle of 24° with the same direction calculated from the family (the indetermination on the observed angles were of the order of ~10° within the family). These values were essentially identical with those observed in the X-ray structure of the bovine protein. The two rotations were in the same direction, bringing the normal to the His planes closer to the α - γ meso direction. It is therefore expected that the *Y*-axis of the magnetic susceptibility tensor would make an angle of 35° with the Fe-pyrrole I nitrogen direction, moving towards the β - δ meso direction, which was in agreement with our observed average value of 21°. In addition, at 25°C and

Fig. 4. Display of the backbone of the final family of structures calculated with PSEUDOREM as a tube with variable radius, proportional to the rmsd of the backbone of each residue. Black indicates β strands and α helices, and the rest of the backbone is shown in light gray. The heme moiety and the side chains of the axial histidines are shown in black as well. The figure has been created with the program MOLMOL (Koradi et al. 1996).

Fig. 5. Experimental and fitted binding curve constructed for the evaluation of the mutant I (E48, E56/A, D60/A) binding constant K by monitoring resonance arising from heme 8-methyl of cyt c .

pH 7.0, in 0.1 mol/L phosphate buffer, the redox potentials are 13 mV for mutant I, 8 mV for mutant II, 15 mV for mutant III, and 5 mV for wild-type cytochrome b_5 , respectively (Wang et al., unpubl.). As is generally known, the observed redox potentials diversity is solely the result of the protein environment of the heme, so mutants that exhibit little changed redox potentials can be considered to lack perturbations of the heme environment (Caffrey and Cusanovich 1994).

From the above discussion, it is concluded reasonably that the mutation of these surface-charged residues do not much alter the overall three-dimensional structure or secondary structure of cyt b_5 . Differences between the interactions of the cyt b_5 mutants with cyt c and those of the wild system are because of electrostatic interaction changes caused by the mutation of the surface-charged key residues. This provides a basis for studying electrostatic effects on interactions and the intrinsic electron transfer process of cyt b_5 and cyt c .

The binding between cyt b_5 and cyt c

Chemical shift variation curves of the heme methyl-8 of cyt c were used to monitor the titration of horse-heart cyt c with the different cyt b_5 mutants. The value of the conditional association constant can be obtained by combining the following equations proposed by María et al. (1996):

$$\delta_o = \frac{\delta_c - \delta_{bc}}{1 + K[b_5]} + \delta_{bc} \quad (2)$$

Equation 2 relates δ_o , the experimentally observed chemical shift at any point in the titration curve, to $[b_5]$, the concentration of free cyt b_5 . Values of δ_c and δ_{bc} can be obtained

directly from the spectra, the value of $[b_5]$ is obtained as described below:

$$[b_5] = \frac{A_b - [b_5]}{K(A_c - A_b + [b_5])} \quad (3)$$

The terms A_c and A_b represent the analytical concentration of cyt c and cyt b_5 , respectively. In equation 3, the binding constant K was initially assumed to obtain $[b_5]$, after which the calculated values of $[b_5]$ and the assumed K value were subsequently used to calculate the value of the observed chemical shift δ_o by substituting these values in equation 2. If the agreement between experimental and calculated binding curves is not acceptable, the process should be started again with a more suitable value of K . The titration and fitting curve of mutant I is shown in Figure 5. The values of the association constant for mutant I-cyt c complex and mutant II-cyt c complex, together with those of mutant III-cyt c complex and wild-type cyt b_5 -cyt c complex reported previously, are listed in Table 2. Throughout the titration, the ionic strength was estimated to vary from 0.01 to 0.03 M for wild-type cyt b_5 -cyt c and from 0.01 to ~0.02 M for the mutants system according to the protein concentrations and the net charges of wild-type cyt b_5 (9-), the triple mutant (6-), the quadruple mutant (5-) and cyt c (10+), respectively (Eley and Moore 1983). The ionic strength dependence of the association constant for the cyt b_5 and cyt c complex is depicted in Figure S2 (shown in the supplemental material), which shows that our wild-type complex association constant for the present condition falls close to the extrapolated values obtained from the values reported previously (María et al. 1996; Mauk et al. 1982).

The association constants of mutant I, mutant II, and mutant III are ~50%, 59%, and 24% of that of wild-type cyt b_5 , respectively, showing clearly the modified residues Glu44, Glu48, Glu56, and Asp60, which were suggested to participate in the formation salt bridges with cyt c in the Northrup and Salemme models, have substantial effect on the complex formation. This indicates that the electrostatic interactions provide important stabilizing factors in the association between cyt b_5 and cyt c . Moreover, after glutamic

Table 2. Association constants of cyt b_5 wild type and mutants with cyt c at pH 7.0 in 1 mM phosphate buffer

Protein	Association constant (M^{-1}) ^a	Percentage (%)
Wild-type Cyt b_5	2.2×10^{4b}	100
Mutant I (E48, E56/A, D60/A)	1.1×10^4	50
Mutant II (E44, E56/A, D60/A)	1.3×10^4	59
Mutant III (E44, E48, E56/A, D60/A)	5.2×10^{3b}	24

^a Errors are estimated to be about 20% of the values.

^b The results obtained from our previous report (Wu et al. 2001).

acid was substituted by alanine, the hydrophobic free energy would increase ~ 0.9 kJ/mol; after aspartic acid was substituted by alanine, the hydrophobic free energy would increase ~ 0.3 kJ/mol (Wang et al., unpubl.). So substituted hydrophobic alanines would provide a more hydrophobic microenvironment of the exposed heme edge, and that should facilitate the association of two proteins. Now the remarkably decreased association constants enhance the idea that electrostatic interactions contribute significantly to the stability of complex formation. At the same time, it is apparent that there is a significant degree of cumulation of the electrostatic contributions to the complex formation in the proposed interface domain. Removal of three proposed salt bridges through the two triple mutations (E44, E56/A, D60/A, or E48, E56/A, D60/A) results in the reduced association constant by $\sim 41\%$ and 50% . Elimination of one more residue (E44, E48, E56/A, D60/A) leads to 76% reduction of the association constant. This indicates that the contributions of the cyt b_5 surface negatively charged residues Glu44, Glu48, Glu56, and Asp60 have a cumulative effect on the stability of cyt c -cyt b_5 complex. At the same time, it demonstrates that the contributions of both Glu44 and Glu48 to the protein association are significant, and the contribution of Glu48 is a little higher than that of Glu44. Previously, we found that Glu44 plays more of a role in the protein mutual association than Glu56 (Sun et al. 1999), which means that the electrostatic contributions of the surface-charged residues to the protein association are different. The above conclusions differ from what Rodgers and Sliger (1991) obtained. These authors concluded that "electrostatics did not provide the main stabilizing factors in the overall association of the protein-protein complex" as the removal of all four proposed salt linkages by mutation (E44Q, E48Q, D60N, DME) decreased the free energy of the cyt b_5 -cyt c complex by only 14% . In fact, according to the following equation: $K = e^{-\Delta G/RT}$, we can convert the binding free energies to the association constants that reflect the electrostatic effects of surface-charged residues' mutations on the complex formation. In Rodgers' and Sliger's experiment, the association constant of cyt b_5 quadruple mutant (E44Q, E48Q, D60N, and DME) with cyt c ($3.3 \times 10^5 \text{ M}^{-1}$) is only 13% of that of wild-type cyt b_5 with cyt c ($2.5 \times 10^6 \text{ M}^{-1}$), greatly decreased values suggest that electrostatic interactions contribute significantly to the stability of the complex formation, which is compatible with our conclusion and in conflict with Rodgers' and Sliger's conclusion, "electrostatics do not appear to contribute significantly to the stability of the complex formation." In addition, Rodgers and Sliger also concluded that "the overall differences in thermodynamic parameters are non-additive." However, the data in Figure 4 of Rodgers' and Sliger's paper do reflect that the effects of multiple mutations have an "additive" (however, "cumulative" is a better word) role on the thermodynamic parameters ΔV and ΔG , which is

also in conflict with their conclusion "These differences were not thermodynamically additive in relation to the single mutations." (Rodgers and Sliger 1991). In fact, the association constant of cyt b_5 mutant (E48Q) with cyt c ($1.25 \times 10^6 \text{ M}^{-1}$), cyt b_5 double-site mutant (E44Q, E48Q) with cyt c ($6.0 \times 10^5 \text{ M}^{-1}$), cyt b_5 quadruple mutant (E44Q, E48Q, D60N, and DME) with cyt c ($3.3 \times 10^5 \text{ M}^{-1}$) is $\sim 50\%$, 24% , and 13% of that of wild-type complex ($2.5 \times 10^6 \text{ M}^{-1}$) in Rodgers' and Sliger's experiments, the comparison of different cyt b_5 mutants' association constants with cyt c further indicate Rodgers' and Sliger's data substantiate our conclusion that the electrostatic effects of surface-charged residues have a cumulative effect on the cyt c -cyt b_5 complex formation.

In fact, after the mutation of these surface-charged residues, the global electrostatic properties of cyt b_5 are affected, causing the charge distribution of the protein to become less asymmetric. Our previous result has demonstrated that the mutant II and mutant III dipole moment through the heme edge was -220D and -134D , respectively, smaller than that of the wild-type protein (-250D) (Ma et al. 1999). The decrease of the dipole moment through the exposed heme edge is one of the factors of lowering the association and electron transfer between two proteins (Rush et al. 1987).

Removal of three residues Glu48, Glu56, and Asp60, which were suggested to participate in the complex formation in the Northrup model, caused the association constant to be reduced by 50% . Previous study has shown the heme propionate on the exposed heme edge participates in electrostatic binding to cyt c (María et al. 1996), such that in the wild-type system the bonding geometry proposed by Northrup, which included Glu48, Glu56, Asp60, and heme propionate of cyt b_5 , indeed occurs in the interaction between cyt b_5 and cyt c in the solution. As to mutant II (E44, E56/A, D60/A), its association constant is close to that of mutant I (E48, E56/A and D60/A), meaning that a docking geometry, which at least includes Glu44, Glu56, and Asp60, may exist in the solution, though slightly differing from the Northrup and Salemme models (Northrup et al. 1993; Salemme 1976).

Materials and methods

Sample isolation and preparation

Trypsin-solubilized bovine liver microsomal cyt b_5 mutants were constructed and expressed in *Escherichia coli* as previously described (Ma et al. 1999).

Horse-heart cyt c (type VI) from Sigma Chemical Company was purified as previously described (Brautigan et al. 1978) and lyophilized once from D_2O before use to exchange all labile protons.

NMR spectroscopy

The ^1H NMR samples were prepared by dissolving ~ 4 mM mutant I (E48, E56/A, and D60/A) in 10 mM phosphate buffer (pH 7.0) in

90% H₂O/10% D₂O, or 99.96% D₂O. 2D DQF-COSY (Derome et al. 1990), TOCSY (Bax and Davis 1985), and TPPI NOESY (Macura et al. 1982) maps both in H₂O and D₂O were acquired on a NMR Bruker DMX600 spectrometer. NOESY maps over a spectral width of 42 ppm and of 14 ppm with mixing times of 56 ms and 100 ms, with a recycle time of 500 ms and 1.2 sec were recorded at 600.13 MHz. COSY and TOCSY maps were acquired over a spectral width of 14 ppm with a recycle time of 1.2 sec and, for the TOCSY maps, spin-lock time 80 ms. 1D NOE experiments on the paramagnetically shifted signals were recorded using the reported methodology (Banci et al. 1989).

Data processing was performed using the standard Bruker software package XWINNMR on a Silicon Graphics workstation. The 2D spectra were analyzed with the aid of the program XEASY (Eccles et al. 1991).

Structure calculation

The majority of the dipolar connectivities were measured from the 100 ms NOESY maps in H₂O at 303 K. Connectivities involving paramagnetically shifted resonances were measured from the 56 ms NOESY in H₂O at the same temperature and from 1D NOE experiments. The volume of NOESY cross peaks was integrated using the elliptical integration routine of XEASY. NOESY cross-peak intensities were translated into interatomic distances following the methodology of the program CALIBA (Güntert et al. 1991). During the course of the structure calculations, stereospecific assignments were obtained and verified with the program GLOMSA (Güntert et al. 1991).

Pseudocontact shifts were employed as additional constraints for the structure calculations. Pseudocontact shifts values were obtained by subtracting the chemical shifts measured for the diamagnetic major form of wild-type bovine cyt *b*₅ from the chemical shifts measured in the oxidized form of cyt *b*₅ mutant (Veitch et al. 1990). No pseudocontact shifts were introduced for the mutated residues and their neighboring residues. The residues contain non-negligible contact shift contributions, such as histidine 39; histidine 63 and the heme also were not used in structural calculations. A total of 190 pseudocontact shift constraints were used (data shown in supplemental material).

Structure calculations were performed with the program PSEUDYANA (Banci et al. 1998), a modified version of the program DYANA (Güntert et al. 1997), adapted to include pseudocontact shifts as additional restraints. The two axial ligands (His39 and His63) were coordinated to the iron atom by additional upper (0.220 nm) and lower (0.190 nm) distance limits from the Nε2 atoms to the central iron atom (Arnesano et al. 1998). After each cycle of structure calculations, the magnetic anisotropy parameters were reevaluated through program FANTASIAN (Banci et al. 1997), and used as input for the following calculation until the final values deviated no more than 5% from the initial ones. Two hundred random structures were annealed in 18,000 steps each and the 35 structures with the lowest target function were included in the family. Finally, the module PSEUDOREM (restrained-energy minimization combined with pseudocontact shifts constraints) (Banci et al. 1997) with the sander module of AMBER (Pearlman et al. 1997) was applied to these 35 structures. Structure calculations and analysis were performed on a Silicon Graphics workstation.

Binding of cyt *b*₅ mutants to cyt *c*

The association constant between cyt *b*₅ mutants and cyt *c* was used to demonstrate the influence of the mutations on the affinity

of two proteins. The titration of cyt *c* at constant concentration with cyt *b*₅ mutant was carried out at 300 K. In comparison, the titration by the mutant and wild-type cyt *b*₅ was done under almost the same experimental condition. Cyt *c* solutions (1 mM) were prepared in 1 mM phosphate buffer (pH 7.0). To keep the concentration of cyt *c* unaffected, cyt *b*₅ was added in solid state directly. The concentration of cyt *b*₅ was varied from 0 to 2.5 mM.

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References

- Arnesano, F., Banci, L., Bertini, I., and Felli, I.C. 1998. The solution structure of oxidized rat microsomal cytochrome *b*₅. *Biochemistry* **37**:173–184.
- Banci, L., Bertini, I., Luchinat, C., Piccioli, M., Scozzafava, A., and Turano, P. 1989. ¹H NOE studies on dicopper(II) dicobalt(II) superoxide dismutase. *Inorg. Chem.* **28**: 4650–4656.
- Banci, L., Bertini, I., Gori, S.G., Romagnoli, A., Turano, P., Cremonini, M.A., Luchinat, C., and Gary, H.B. 1997. The pseudocontact shifts as constraints for energy minimization and molecular dynamic calculations on solution structures of paramagnetic metalloproteins. *Proteins* **29**: 68–76.
- Banci, L., Bertini, I., Cremonini, M.A., Gori, S.G., Luchinat, C., Wüthrich, K., and Güntert, P. 1998. PSEUDYANA for NMR structure calculation of paramagnetic metalloproteins using torsion angle molecular dynamics. *J. Biomol. NMR* **12**: 553–557.
- Banci, L., Bertini, I., Rosato, A., and Scacchier, S. 2000. Solution structure of oxidized microsomal rabbit cytochrome *b*₅. *Eur. J. Biochem.* **267**: 755–766.
- Bax, A. and Davis, D.G. 1985. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **65**: 355–360.
- Brautigan, D.L., Miller, S.F., and Margoliash, E. 1978. Mitochondrial cytochrome *c*: preparation and activity of native and chemically modified cytochromes *c*. *Methods Enzymol.* **53D**: 128–164.
- Burch, A.M., Rigby, S.E.J., Funk, W.D., Macgillivray, R.T.A., Mauk, M.R., Mauk, A.G., and Moore, G.R. 1990. NMR characterization of surface interactions in the cytochrome *b*₅-cytochrome *c* complex. *Science* **247**: 831–833.
- Caffrey, M.S. and Cusanovich, M.A. 1994. Site-specific mutagenesis studies of cytochrome *c*. *BBA*. **1187**:277.
- Dangi, B., Sarma, S., Yan, C., Banville, D.L., and Guiles, R.D. 1998. The origin of difference in the physical properties of the equilibrium forms of cytochrome *b*₅ through high-resolution NMR structures and backbone dynamic analyses. *Biochemistry* **37**: 8289–8302.
- Derome, A.E. and Williamson, M.P. 1990. Rapid-pulsing artifacts in double-quantum-filtered cosy. *J. Magn. Reson.* **88**: 177–185.
- Dreyer, J.L. 1984. Electron transfer in biological system: An overview. *Experientia* **40**: 653–675.
- Durley, R.C.E. and Mathews, F.S. 1996. Refinement and structural analysis of bovine cytochrome *b*₅ at 1.5 Å resolution. *Acta. Crystallog. sect. D* **52**: 65–72.
- Eccles, C., Güntert, P., Billeter, M., and Wüthrich, K. 1991. Efficient analysis of protein 2D NMR spectra using the software package EASY. *J. Biomol. NMR* **1**: 111–130.
- Eley, E.G.S. and Moore, G.R. 1983. ¹H-NMR investigation of the interaction between cytochrome *c* and cytochrome *b*₅. *Biochem. J.* **215**: 11–21.
- Güntert, P., Braun, W., and Wüthrich, K. 1991. Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS and GLOMSA. *J. Mol. Biol.* **217**: 517–530.
- Güntert, P., Mumenthaler, C., and Wüthrich, K. 1997. Torsion angle dynamics

- for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* **273**: 283–298.
- Hom, K., Ma, Q.F., Wolfe, G., Zhang, H., Storch, E.M., Daggett, V., Basus, V.J., and Waskell, L. 2000. NMR studies of the association of cyt *b₅* with cyt *c*. *Biochemistry* **39**: 14025–14039.
- Koradi, R., Billeter, M., and Wüthrich, K. 1996. MOLMOL: A program for display and analysis of macromolecular structure. *J. Mol. Graphics* **14**: 51–55.
- Kurland, R.J. and McGarvey, B.R. 1970. Isotropic NMR shifts in transition metal complexes: calculation of the Fermi contact and pseudocontact terms. *J. Magn. Reson.* **2**: 286–301.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**: 283–291.
- Laskowski, R.A., Rullmann, J.A.C., MacArthur, M.W., Kaptein, R., and Thornton, J.M. 1996. AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* **8**: 477–486.
- Ma, D.J., Wu, Y.B., Qian, C.M., Tang, W.X., Wang, Y.H., Wang, W.H., Lu, J.X., Xie, Y., and Huang, Z.X. 1999. Effects of some charged amino acid mutations on the electron self-exchange kinetics of cytochrome *b₅*. *Inorg. Chem.* **38**: 5749–5754.
- Macura, S., Wüthrich, K., and Ernst, R.R. 1982. The relevance of the cross-peaks in two-dimensional NOE experiments of macromolecules. *J. Magn. Reson.* **47**: 351–357.
- Marcus, R.A. and Sutin, N. 1985. Electron transfers in chemistry and biology. *Biochim. Biophys. Acta.* **811**: 265–322.
- María, J., Marañón, R., Qiu, F., Stark, R.E., White, S.P., Zhang, X.J., Foundling, S.I., Rodríguez, V., Schilling, C.L., Bunce, R.A., and Rivera, M. 1996. ¹³C NMR spectroscopic and X-ray crystallographic study of the role played by mitochondrial cytochrome *b₅* heme propinates in the electrostatic binding to cytochrome *c*. *Biochemistry* **35**: 16378–16390.
- Mauk, A.G., Mauk, M.R., Moore, G.R., and Northrup, S.H. 1995. Experimental and theoretical analysis of the interaction between cytochrome *c* and cytochrome *b₅*. *J. Bioenerg. Biomemb.* **27**: 311–330.
- Mauk, M.R., Reid, L.S., and Mauk, A.G. 1982. Spectrophotometer analysis of the interaction between cytochrome *b₅* and cytochrome *c*. *Biochemistry* **21**: 1843–1846.
- Muskett, F.W., Kelly, G.P., and Whitford, D. 1996. The solution structure of bovine ferricytochrome *b₅* determined using heteronuclear NMR methods. *J. Mol. Biol.* **258**: 172–189.
- Northrup, S.H., Thomasson, K.A., Miller, C.M., Barker, B.D., Eltis, L.D., Guillemette, J.G., Inglis, S.C., and Mauk, A.G. 1993. Effects of charged amino acid mutations on the biomolecular kinetics of reduction of the yeast iso-ferricytochrome *c* by bovine ferrocyclochrome *b₅*. *Biochemistry* **32**: 6613–6623.
- Pearlman, D.A., Case, D.A., Caldwell, J.W., Ross, W.S., Cheatham, T.E., Ferguson, D.M., Seibel, G.L., Singh, U.C., Weiner, P.K., and Kollman, P.A. 1997. *AMBER 5.0* University of California, San Francisco.
- Rodgers, K.K., Prochapsky, T.C., and Sligar, S.G. 1988. Probing the mechanisms of macromolecular recognition: The cytochrome *b₅*-cytochrome *c* complex. *Science* **240**: 1657–1659.
- Rodgers, K.K. and Sligar, S.G. 1991. Mapping electrostatic interactions in macromolecular associations. *J. Mol. Biol.* **221**: 1453–1460.
- Rush, J.D., Lan, J., and Koppenol, W.H. 1987. Effect of dipole moment on the ionic strength dependence of electron-transfer reactions of cytochrome *c*. *J. Am. Chem. Soc.* **109**: 2679–2682.
- Salemme, F.R. 1976. A hypothetical structure for an intermolecular electron transfer complex of cytochrome *c* and cytochrome *b₅*. *J. Mol. Biol.* **102**: 563–568.
- Shokhirev, N.V. and Walker, F.A. 1998. Co- and counter-rotation of magnetic axes and axial ligands in low-spin ferriheme systems. *J. Am. Chem. Soc.* **120**: 981–990.
- Storch, E.M. and Daggett, V. 1995. Molecular dynamics simulation of cytochrome *b₅*: Implication for protein-protein recognition. *Biochemistry* **34**: 9782–9693.
- Sun, Y.L., Wang, Y.H., Yan, M.M., Sun, B.Y., Xie, Y., Huang, Z.X., Jiang, S.K., and Wu, H.M. 1999. Structure, interaction and electron transfer between cytochrome *b₅*, its E44A and/or E56A mutants and cytochrome *c*. *J. Mol. Biol.* **285**: 347–359.
- Veitch, N.C., Whitford, D., and Williams, R.J.P. 1990. An analysis of pseudo-contact shifts and their relationship to structural features of the redox states of cytochrome *b₅*. *FEBS Letters* **269**: 297–304.
- Wu, Y.B., Wang, Y.H., Qian, C.M., Lu, J., Li, E.C., Wang, W., Lu, J.X., Xie, Y., Zhu, D.X., Huang, Z.X., and Tang, W.X. 2001. Solution structure of cytochrome *b₅* mutant (E44/48/56A/D60/A) and its interaction with cytochrome *c*. *Eur. J. Biochem.* **268**: 1620–1630.