Solution structure of cytochrome $b_5$ mutant (E44/A48/56A/D60A) and its interaction with cytochrome $c$

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Using 1617 meaningful NOEs with 188 pseudocontact shifts, a family of 35 conformers of oxidized bovine microsomal cytochrome $b_5$ mutant (E44/A48/56A/D60A) has been obtained and is characterized by good resolution (rmsd to the mean structure are $0.047 \pm 0.007$ nm and $0.095 \pm 0.008$ nm for backbone and heavy atoms, respectively). The solution structure of the mutant, when compared with the X-ray structure of wild-type cytochrome $b_5$, has no significant changes in the whole folding and secondary structure. The binding between cytochrome $b_5$ and cytochrome $c$ shows that the association constant of the mutant–cytochrome $c$ complex is much lower than the one for wild-type complex (2.2 $\times 10^4$ M$^{-1}$ vs. 5.1 $\times 10^4$ M$^{-1}$).

The result suggests the four acidic residues have substantial effects on the formation of the complex between cytochrome $b_5$ and cytochrome $c$, and therefore it is concluded reasonably that the electrostatic interaction plays an important role in maintaining the stability and specificity of the complex formed. The competition between the ferricytochrome $b_5$ mutant and [Cr(oxalate)$_3$]$^{3+}$ for ferricytochrome $c$ shows that site III of cytochrome $c$, which is a strong binding site to wild-type cytochrome $b_5$, still binds to the mutant with relatively weaker strength. Our results indicate that certain bonding geometries do occur in the interaction between the present mutant and cytochrome $c$ and these geometries, which should be quite different from the ones of the Salemme and Northrup models.

**Keywords**: cytochrome $b_5$; cytochrome $c$; mutagenesis; solution structure; electrostatic interaction.

Biological electron transfer (ET) reactions play an essential role in numerous important biological processes such as photosynthesis, oxidative phosphorylation, and xenobiotic processing [1,2]. Intermolecular electron transfer reactions involve highly specific interactions between the redox components via noncovalent bonding motifs of the protein surfaces. An excellent model system is the interaction between cytochrome $b_5$ (cyt $b_5$) and cytochrome $c$ (cyt $c$), which are regarded as physiological redox partners in vivo [3]. The experimental and theoretical investigation of the interaction of such two proteins performed over a period of 20 years has provided a considerable insight into the manner in which these proteins recognize and bind to each other [4–7], and the initial characterization of the nature of this complex [8]. However, the question as to the surface area of cyt $b_5$ involved in the recognition of cyt $c$ and the forces contributing significantly to the stability of the complex is still open. Based on the known three-dimensional structures of cyt $b_5$ and cyt $c$, attention was first directed to the interaction of the two proteins by the computer graphics modeling study of Salemme [9], in which he proposed that complementary electrostatic interactions would result in formation of a 1 : 1 cyt $b_5$–cyt $c$ complex of appropriate geometry for efficient electron transfer. The model obtained by Northrup et al. using the Brownian dynamic simulations calculations [10] predicted that the two proteins approach each other with two different docking geometries and a subsequent refinement of the two protein complexes showed that the final complexes were significantly different from those originally proposed [8]. This indicates that electrostatic interaction at the molecular interface results in flexible association complexes. However, the utilization of high-pressure techniques coupled with site-directed mutagenesis demonstrated that electrostatic force did not provide the main stabilizing factors in the overall association of this protein-protein complex [11]. It is clear that there are different standpoints concerning the roles of the electrostatic interactions involved in the formation of the electron transfer complex.

In order to investigate the role of the electrostatic interactions in the cyt $b_5$–cyt $c$ complex formation, we prepared a cyt $b_5$ mutant (E44/A48/56A/D60A), in which all the negatively charged amino-acid residues involved in the complex formation according to the two models were altered to the hydrophobic alanine by site-directed mutagenesis [12]. The high quality three-dimensional solution structure of the present mutant determined by means of NMR allows us a further understanding of the functional
properties of the present mutant. Our experimental results indicate that electrostatic interactions might contribute considerably to macromolecular associations. The influence of the four acidic residues on recognition of two proteins was also studied using the competitive paramagnetic difference spectroscopy method [13].

**MATERIALS AND METHODS**

**Sample preparation**

Trypsin-solubilized bovine liver microsomal cyt b5 mutant (E44/48/56A/D60A) was prepared as described before [12]. Horse heart cyt c (type VI) from the Sigma Chemical Co. was purified as previously described [14] and lyophilized once from D2O. K3[Cr(oxalate)3] was synthesized by the approach reported previously [15].

**NMR spectroscopy**

1H NMR spectra for solution structure determination were acquired on a Bruker DMX600 spectrometer. Samples contained ≈6 mm protein, in 10 mm phosphate buffer (pH 7.0) in 90% H2O/10% D2O or 99.96% D2O. To detect connectivities among hyperfine-shifted signals, 2D NOESY experiments [16,17] were acquired with a spectral width of 56 p.p.m. in both frequency dimensions and a mixing time of 60 ms. To optimize the detection of connectivities in the diamagnetic region (−3 to 13 p.p.m.), 2D NOESY and TOCSY [18] spectra were acquired with mixing times of 100 ms, 200 ms, and a spin-lock time of 50, 80 ms in H2O and D2O, respectively. DQF-COSY [19,20] spectra were recorded in H2O and D2O. In spectra acquired, the water signal was suppressed through the presaturation sequence. Quadrature detection was achieved using the TPPI method [17]. All data consisted of 4 K data points in the acquisition dimension and of 512 experiments in the second dimension. The 1D 1H NOE experiments were performed irradiating at −13.4, 12.7 p.p.m. following the standard procedure [21,22]. Raw data of 2D spectra were weighted with a squared cosine function, zero-filled, and Fourier-transformed to obtain a final matrix of 4 K × 4 K data points. The above spectra were collected at 303 K.

1H NMR spectra for studies of interactions between cyt b5 and cyt c were recorded on the Bruker AM 500 spectrometer. Chemical shifts for all spectra were referenced to 1,4-dioxane at 3.74 p.p.m. All spectra were processed using the xWINNMR software on the SGI Indy workstation. The 2D spectra were analyzed with the aid of the program XEASY [23].

**Structure calculations**

The volumes of NOESY cross peaks between assigned resonances were measured using the elliptical integration routine of the program XEASY [23]. Intensities of dipolar connectivities were converted into upper distance limits, to be used as input for structure calculations, through the program CALIBA [24]. 1D NOEs involving paramagnetic signals were converted into relatively long upper distance limits of equal length (0.550 nm). During the course of the structure calculations, stereospecific assignments were obtained and verified with the program GLOMSA [24].

Pseudocontact shifts were employed as additional constraints for the structure calculations and given by [25]:

$$\delta^\text{pcs} = \frac{1}{12\pi r_i^2} \left[ \Delta \chi_{\text{m}} (3n_i^2 - 1) + \frac{3}{2} \chi_{\text{m}} (l_i^2 - m_i^2) \right]$$  

where \(\Delta \chi_{\text{m}}\) and \(\Delta \chi_{\text{m}}\) are the axial and rhombic magnetic susceptibility anisotropies, \(r_i\) the length of the nuclei \(i\) from the metal ion, and \(l_i, m_i, n_i\) 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. Pseudocontact shifts values were obtained by subtracting from the chemical shifts measured in the oxidized form of cyt b5 mutant the chemical shifts measured for the diamagnetic major form of wild-type bovine cyt b5 [26,27]. No pseudocontact shifts were introduced for the residues that were mutated, for their neighboring residues and for the heme and the axial heme ligands (His39 and His63) that experienced a non-negligible contact shift. A total of 188 pseudocontact shift constraints were used (data not shown).

Structure calculations were performed with the program PSEUDYANA [28], a modified version of the program DYANA [29] 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 Ni2 atoms to the central iron atom [30]. After each cycle of structure calculations, the magnetic anisotropy parameters were re-evaluated through the program FANTASIAN [31,32] 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 15 000 steps each and the 35 structures with the lowest target function were included in the family. Finally, the module PSEUDOREM [32] (restrained energy minimization combined with pseudocontact shifts constraints) with the sander module of AMBER [33] was applied to these 35 structures. Of the resulting structures an average structure was calculated and underwent another REM.

The quality of the structure was determined using the programs PROCHECK [34] and PROCHECK-NMR [35]. Structure calculations and analysis were performed on SGI Indigo-2 and ORIGIN200.

**Interaction and recognition between the cyt b5 mutant and cyt c**

The association constant between cyt b5 and cyt c was used to demonstrate the influence of the mutations on the binding of the two proteins. The titration of cyt c at constant concentration with cyt b5 was carried out at 300 K. In comparison, the titration by wild-type and the mutant was done under almost the same experimental condition. Cyt c solutions (1 mm) were prepared in 1 mm phosphate buffer (pH 7.0). To maintain the concentration of cyt c, cyt b5 was added in solid state directly. The concentration of cyt b5 was varied from 0 to 2.5 mm. The competition between the mutant and [Cr(oxalate)]2+ for ferriicytochrome c was studied by way of competitive paramagnetic difference
spectra, which were obtained as previously described [13]. Samples for paramagnetic difference spectra contained 2–4 mM for the mutant and 4 mM for cyt c.

RESULTS AND DISCUSSION

Sequence-specific assignment

Extensive lists of assignments for oxidized cyt b₅ from different organisms are available in the literature for both the diamagnetic part of the protein [26,36–38] and hyperfine-shifted signals [39–42]. Under our experimental conditions, there are two forms of solution due to two conformations of the heme ring differing by a 180° rotation around the α–γ axis [39]; the major form constituting roughly 90% of the population is our aim. In this work, the available assignments were extended to 77% of the total protons (including the ones of the heme) and all residues but Ala3 (data not shown). The present assignment is consistent with two sets of assignments reported for oxidized bovine cyt b₅ [26,36]; 46 new assignments have been obtained. The assignment of Ser18, which locates the edge of an external loop, is tentative because there are not enough NOEs to affirm its assignment.

Secondary structure

Figure 1 shows the short- and medium-range NOEs observed for the backbone and β protons. In general, helical structures can be identified by the high number of sequential and medium range connectivities such as

\[
d_{\text{HN}}(i,i+1), d_{\text{HN}}(i,i+3), d_{\text{NH}}(i,i+4), \text{ and } d_{\text{off}}(i,i+3), \]

while β strands are expected to give strong \(d_{\text{oN}}\) sequential and intraresidue connectives and weak \(d_{\text{IN}}\) connectivities. Five elements of helical secondary structure can be identified by NOE pattern. They involve residue 9–15 (α1), 32–39 (α2), 43–49 (α3), 55–62 (α4) and 64–75 (α5), similar to the characteristic secondary structural elements present in all cyt b₅ crystal and solution structures. The pattern of long-range NOEs (data not shown) 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.

Solution structure determination

A total of 1994 NOESY experimental constraints, most taken from the mixing time of 100 ms NOESY in H₂O, were obtained and transformed into upper distance limits with the program CALIBA [24]. In the present structure, the calibration of observed intensities was selected to be inversely proportional to the sixth power of the proton–proton distances for all calibration classes of NOE constraints. Among the total 1994 distance limits, 1617 constraints were found to be meaningful (corresponding to 24.3 or 19.7 constraints per residue, respectively) and used in the structure calculations together with 188 pseudocontact shifts constraints. The number of experimentally meaningful NOEs per residue is demonstrated in Fig. 2B. A total of 28 stereospecific assignments were obtained through the program GLOMIA [24].

The 35 conformers obtained from PSEUDYANA with the lowest target function constituted the final family, which has rmsd values (hereafter, unless specified, rmsd value are calculated for residues 5–82) to the mean structure of 0.048 ± 0.006 nm for the backbone and 0.092 ± 0.008 nm for the heavy atoms. The average total target function value obtained through the program pseudorenm [24]. The resulting family has rmsd values of 0.047 ± 0.008 nm and 0.095 ± 0.008 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 Fig. 2A. The average penalty function of 43.59 kJ·mol⁻¹ (30.11–59.59 kJ·mol⁻¹) corresponds to a target function values of 0.0033 nm² (0.0022–0.0045 nm²) while 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 [34] and PROCHECKNMR [35]. For the energy-minimized mean structure, the following residues were found to form α helices: 9–14, 33–35, 43–49, 55–61 and 65–73. Assignment of all helices identified by NOEs pattern was achieved through the program PROCHECK except that helix α2 has a different length. The helix α2 (residues from 32 to 39) cannot be classified as a canonical α helix [43] for solution structure wild-type bovine and rat cyt b₅ [44,45]. The short segment involving the C-terminus...
residues 82–84, which was part of the helix o6 (82–87) present in crystal, also showed a helical structure in some conformations of the family. Four segments of β secondary structures were identified by procheck for residues 6–7, 75–79, 28–31 and 20–25 commonly referred to as strands β1–β4, β6 (16–17), which exists in X-ray structure (PDB accession no. 1cyo) [46], also appeared while β5 involving 51–54 was not found by the program procheck as reported [45]. In the average-minimized structure, 90.3% of the residues were found in the most favored regions of the Ramachandran plot, 6.9% in the allowed regions and 2.8% in a generously allowed region. No residues were found in the disallowed regions.

Table 1 reports calculation statistics and the structure quality analysis for the solution structure. No distance violations were bigger than 0.3 and no forbidden Van der Waal’s contacts observed in both the family and the average structure. The above results indicated the good quality of the present solution structure. Figure 3 shows the structure of the family represented as a tube whose radius is proportional to the rmsd of the family.

**Magnetic susceptibility tensor**

The final $\Delta X_{ax}$ and $\Delta X_{rh}$ values were $2.71 \times 10^{-32}$ and $-1.07 \times 10^{-32}$ m$^3$, respectively. The principal z axis of the magnetic anisotropy tensor formed an angle about 6° towards the perpendicular to the heme plane whereas the x axis of the tensor made an angle of about 25° with the α-γ meso direction. The $\Delta X_{ax}$ and $\Delta X_{rh}$ were in good agreement with those reported for solution structures of rat and rabbit cyt bs [30,47].

It is known that the orientation of the in-plane axes of the magnetic susceptibility tensor is essentially dependent on the relative arrangement of the iron axial ligands [48,49], i.e. in the present case, on the orientation of the imidazole planes of His39 and His63. In all the 35 conformations of the family, the normal to the plane of His39 made an angle of 44° with the Fe-pyrrole I nitrogen direction, while the normal to the plane of His63 made an angle of 22° with the same direction calculated from the family (the indetermination on the observed angles were of the order of about 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 [49] that the y axis of the magnetic susceptibility tensor would make an angle of 33° with the Fe-pyrrole I nitrogen direction, moving towards the β-δ meso direction, which was in agreement with our observed average value of 20°.

Because the mutations took place near His39 and His63, it is of concern to us whether the rings of His39 and/or His63 had reoriented or not. When we superimposed all heavy atoms of heme, the rmsd values of all heavy atoms of His39 and His63 between the average energy-minimized solution structure and the X-ray structure were 0.026 nm and 0.045 nm, respectively. This showed that the orientation of the two imidazoles was not appreciably perturbed by the mutations occurring in the polypeptide chain. Furthermore, our studies showed [50] that the reduction potential of the present mutant (15 mV) was not significantly different from that of the wild-type (4.5 mV). Therefore, we conclude that mutagenesis at the protein surface causes no significant changes in the redox potential of the mutant proteins.

**Comparison with wild-type cyt bs**

As the present structure of this mutant is characterized by high resolution, it is meaningful to compare it with those of wild-type cyt bs. A crystal structure of the bovine oxidized form was obtained (PDB accession no. 3b5c) [43], which has been recently refined to 1.5 Å resolution (PDB accession no. 1cyo) [46]. The ribbon diagrams of the average energy-minimized structure of the mutant and the X-ray crystallographic structure of the wild-type (PDB accession no. 1cyo) are shown in Fig. 4, together with a stereo view of the superimposed structures by superimposing all heavy atoms (except the side chains of residues 44, 48, 56 and 60) for residues 5–82. The rmsd between the two structures are 0.079 nm and 0.150 nm for the backbone atoms and heavy atoms, respectively. As can be seen from Fig. 4, the global folds of the two structures remain similar and most of the parts superimpose quite well. This indicates that the present structure is quite similar to the wild-type X-ray structure. The high-resolution solution structure of wild-type rat cyt bs, which has a 94% homology with the wild-type bovine cyt bs, is available (PDB accession no. 1aw3) [30]. The rmsd between solution structures of rat cyt bs and the
The binding between cyt b\textsubscript{5} and cyt c 

Resonances arising from the heme in cyt c were used to monitor the titration of horse heart cyt c with the present mutant and wild-type cyt b\textsubscript{5}. The value of the conditional binding constant can be obtained with the equation proposed by Rivera \textit{et al.} [51]:

\[
\delta_o = \left(\frac{\theta_o}{1 + K[b_o]} + \delta_{bc}\right)
\]

Eqn (2) relates \(\delta_o\), the experimentally observed chemical shift at any point in the titration curve, to \([b_o]\), the corresponding free concentration of cyt b\textsubscript{5}. Values of \(\delta_o\) will be known accurately, leaving \(\delta_{bc}\), the chemical shift of fully complexed cyt c and K, the binding constant to be determined by the regression analysis.

The value of \([b_o]\) is obtained as described below [51]:

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[b_o] = \frac{A_o - [b_o]}{K(A_o - A_c + [b_o])}
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The terms \(A_o\) and \(A_c\) represent the analytical concentration of cyt c and cyt b\textsubscript{5}, respectively. The binding constant \(K\) was initially assumed to obtain \([b_o]\). 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 of cyt c with cyt b\textsubscript{5} shows that some of the heme resonances shift as the fraction of cyt b\textsubscript{5} increases. The titration curve shown in Fig. 5 was constructed by monitoring resonance arising from the heme-8 methyl of cyt c. Cyt c solutions (1 mM) were prepared in 1 mM phosphate buffer (pH 7.0) while the concentration of cyt b\textsubscript{5} mutant is 0.090 nm for the backbone atoms, which clearly shows that the two three-dimensional solution structures from the two different sources are indeed similar.

From the above discussion, it is concluded that the mutation of these surface surrounding residues does not much alter the overall three-dimensional structure or secondary structure. Differences between the interaction of the mutant cyt b\textsubscript{5} with cyt c and that of the wild-type cyt b\textsubscript{5} with cyt c are due to electrostatic interaction changes caused by the mutation of the four key residues. This provides a basis for studying electrostatic effects on interactions and the intrinsic electron transfer process of cyt b\textsubscript{5} and cyt c.

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<table>
<thead>
<tr>
<th>RMS violations per experimental distance constraint (nm) (^a)</th>
<th>Average over the family</th>
<th>Mean structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-residue (350)</td>
<td>0.0017 ± 0.0002</td>
<td>0.0016</td>
</tr>
<tr>
<td>Sequential (370)</td>
<td>0.0013 ± 0.0003</td>
<td>0.0015</td>
</tr>
<tr>
<td>Medium range (^b)</td>
<td>0.0011 ± 0.0002</td>
<td>0.0013</td>
</tr>
<tr>
<td>Long range (544)</td>
<td>0.0009 ± 0.0002</td>
<td>0.0005</td>
</tr>
<tr>
<td>Total (1617)</td>
<td>0.0013 ± 0.0001</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

Average number of violations per structure:

| Intra-residue                     | 10.77 ± 2.40 | 11  |
| Sequential                       | 8.43 ± 2.44  | 8   |
| Medium range \(^b\)              | 6.20 ± 2.24  | 7   |
| Long range                       | 5.31 ± 1.85  | 4   |
| Total                            | 31.3 ± 5.12  | 42  |
| Violations larger than 0.3 Å     | 0.00 ± 0.00  | 0   |
| Violations between 0.1–0.3 Å     | 5.91 ± 1.89  | 8   |

Target function (nm\(^2\))  

| 0.0033 ± 0.0005 | 0.0034 |

AMBER force field average total energy (kJ·mol\(^{-1}\))  

| -4088 ± 556 | -4863 |

Structure precision (nm) \(^c\)

| Backbone | 0.047 ± 0.007 | –    |
| All heavy atoms | 0.095 ± 0.008 | –    |

Structure analysis \(^d\)

| % of residues in most favoured regions | 79.0 ± 3.5 | 90.3 |
| % of residues in additionally allowed regions | 79.0 ± 3.5 | 90.3 |
| % of residues in generously allowed regions | 17.8 ± 3.9 | 6.9  |
| % of residues in disallowed regions | 2.1 ± 1.5  | 2.8  |
| No. of bad contacts/100 residues | 1.1 ± 1.1  | 0.0  |
| H-bond energy (kJ·mol\(^{-1}\)) | 0.03 | 0.0  |
| Overall G-factor | -0.16 ± 0.04 | - 0.15 |

\(^a\) The number of meaningful constraints for each class is reported in parenthesis. \(^b\) 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 prochecknmr were used to check the overall quality of the structure and Gly and Pro are excluded from the Ramachandran analysis. For the procheck statistic, less than 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 larger than –0.5 are expected for a good quality structure.
was varied from 0 to 2.5 mM. Throughout the titration the ionic strength varied from 0.01 to 0.03 M for wild-type cyt b5–cyt c and from 0.01 to 0.02 M for the mutant–cyt c according to the protein concentrations and the net charges of cyt b5 (9−), the mutant (5−) and cyt c (10+) [13], respectively. The results summarized in Fig. 5 show that the values of the conditional binding constant for the formation of the wild-type complex and the mutant–cyt c complex are $2.2 \times 10^7$ M$^{-1}$ and $5.1 \times 10^4$ M$^{-1}$, respectively. The ionic strength dependence of the binding constant for the cyt b5 and cyt c complex is depicted in Fig. 6, showing that the binding constant for the present condition falls close to the extrapolated values obtained from the values reported previously.

The value of the binding constant of cyt b5 and cyt c reported before is relatively lower [13]. There could be many reasons for the difference in conditional dissociation constant between that and the present work, e.g. as differences in ionic strength or composition.

The fact that the binding constant for the mutant is only about 23% of that for wild-type shows clearly the modified residues have a substantial effect on the complex formation, and indicates that the electrostatic interactions played an important stabilizing factor in the association of the cyt b5 and cyt c complex. However, in 1991 Rodgers & Sligar [11] discovered that the removal of all four proposed salt-linkages by mutation (E44Q, E48Q, D60N, and the dimethyl ester of heme propionates) decreased the free energy of the cyt b5–cyt c complex by only 14% assuming the same complex orientation as the wild-type cyt b5–cyt c association. The authors concluded that electrostatic interactions did not contribute significantly to the stability of complex formation. In their experiment, the four negatively charged groups were altered to their respective amide analogs. More likely, these amide groups introduced could serve as potential hydrogen-bond donors/acceptors with the lysine residues or other polar residues of cyt c. The roles of these possible hydrogen-bonds might compensate the reduction of the thermodynamic contributions of electrostatic interactions in associations [11]. In our experiment, alteration of the four negative residues (E44, E48, E56 and D60) to alanines formed no new hydrogen bonds and the decreased value of binding constant indicated that the contributions of possible hydrophobic interactions of alanines seemed to be small. Greatly decreased values suggested that electrostatic interactions contributed significantly to the stability of the complex formation.

The charge distribution is important for understanding the interactions between the two mentioned cytochromes. As the charge distribution of the present mutant becomes less asymmetric and the component of the dipole moment through the exposed heme edge decreases markedly from $-250$ D (wild-type) to $-134$ D [12] after the mutation of E44, E56, E56 and D60 by alanine, it is understandable that the binding strength of the mutant and cyt c is smaller than that of the wild-type complex.

The relationship between the protein binding and electron transfer (ET) is still unclear up to now [53]. Our studies show that the ET constant of the present mutant–cyt c is about 41% of the one of the wild-type system ($1.98 \times 10^7$ M$^{-1}$s$^{-1}$ vs. $4.86 \times 10^5$ M$^{-1}$s$^{-1}$ at 288 K, pH = 7.0, I = 150 mM) [50]. Although the conditions of binding and ET experiments are not identical, it is still meaningful to compare the values of the mutant–cyt c relative to wild-type for the binding and ET constants. It is interesting to note that the former is much smaller than the latter (23% vs. 41%). The results show that it is a question of whether the protein binding study can precisely describe the precursor formation and path of the ET, although the mutation of the four key residues indeed affects significantly both ET and binding. The binding studies and ET focus on the stable protein–protein complex and kinetic process, respectively, and there is no necessity that the most stable binding geometry is the best ET site.

**Competition between ferricytochrome b5 mutant and [Cr(oxalate)$_3$]$^{3-}$ for ferricytochrome c**

One NMR approach to determine interprotein interaction regions is to use the competitive paramagnetic difference spectroscopy method introduced by Eley & Moore [13]. Figure 7 shows the paramagnetic difference spectra of relaxation reagent [Cr(oxalate)$_3$]$^{3-}$ binding to (a) 4 mM cyt c, (b) the mixture of 4 mM cyt c and 4 mM cyt b5 mutant, and (c) 2 mM cyt b5 mutant.

Binding of relaxation reagent [Cr(oxalate)$_3$]$^{3-}$ to ferricytochrome c was extensively investigated by NMR and it was confirmed that there are three anion-binding sites close to the exposed heme edge of cyt c [13]. The important binding residues in the sites are Lys86 and Lys87 (site I), Lys25 and Lys27 (site II), and Lys13 and Lys72 (site III); site III has a relatively higher affinity than sites II and I. As shown in the Fig. 7A, the residues, which are close to these binding sites and whose resonances can be broadened when the paramagnetic competitive reagent [Cr(oxalate)$_3$]$^{3-}$ binds to these binding sites, are: Ile85, Ala83, Phe82 and Ile81 (site III); Thr19 and His26 (site II); and Met65 and Thr89 (site I). The previous conclusion that Thr28 is affected by the binding of the reagent at Lys79 and site II is consistent with our result obtained from Fig. 7A.
Addition of $[\text{Cr(oxalate)}_3]^{3-}$ to ferricytochrome $b_5$ mutant (shown in Fig. 7C) caused the NMR spectrum only a few specific perturbations, and indicated some weak binding. Some disturbed resonances in cyt $b_5$ difference spectrum were assigned as Val4, Lys5 and Lys19 and indicated that $[\text{Cr(oxalate)}_3]^{3-}$ might bind to some surfaces regions of the cyt $b_5$ mutant, which were far away from the surroundings of its heme.

In Fig. 7B, resonances of Thr19 and His26 (site II), Met65 and Thr89 (site I) and Thr28 are all present. The result indicates that site II, which is protected to some extent in the mixture of wild-type cyt $b_5$ and cyt $c$ [13], and site I are unprotected by the complex formation and occupied by the paramagnetic competitive reagent $[\text{Cr(oxalate)}_3]^{3-}$. The resonances of Ile81 and Ala83 are missing in Fig. 7B, just as in the mixture of wild-type cyt $b_5$ and cyt $c$, while the resonances of Ile85 and Phe82 still remain, and their intensities have dropped a little compared with those in Fig. 7A, but are slightly higher than those in mixture of wild-type cyt $b_5$ and cyt $c$. This indicates that site III, which is a strong binding site to wild-type cyt $b_5$, still binds to cyt $b_5$ mutant, although this binding has been weakened to some degree. From another spectrum obtained under the same conditions as in Fig. 7B except using double concentration of $[\text{Cr(oxalate)}_3]^{3-}$ (data not shown), the resonances of Ile81 and Ala83 now appeared, but their intensities were still lower than those observed in mixture of wild-type cyt $b_5$ and cyt $c$. The result implied that $[\text{Cr(oxalate)}_3]^{3-}$ had a higher affinity for site III with increasing concentration. It is therefore concluded that the binding of cyt $b_5$ mutant and cyt $c$ was not as strong as that of wild-type cyt $b_5$ and cyt $c$ and this is in agreement with the reduction of association constant.

Salemme [9] proposed a hypothetical electron transfer model between cyt $b_5$ and cyt $c$ on the supposition that a complex should be formed between the ε-amino groups of...
Lys13, Lys27, Lys72 and Lys79 of cyt c and the carboxyl group of Glu48, Glu44, Asp60 and the exposed heme propionate of cyt b5, respectively. Based on Brownian dynamics simulations in conjunction with kinetic measurements, Northrup et al. [10] proposed that the most frequently encountered docking geometries involved hydrogen bonding interactions of the following residues (cyt b5±cyt c): Glu48±Lys13, Glu56±Lys87, Asp60±Lys86, and heme±Tml72 with an average electrostatic energy of 213.0 kcal·mol$^{-1}$. The electrostatic stabilization energy of another less stable mode of interaction, which was identical with what was proposed by Salemme, was 26.4 kcal·mol$^{-1}$. When Glu44, Glu48, Glu56 and Asp60 were mutated into alanine, it could be predicted that the salt bridges formed by these residues would break. Thus, the most favorable bonding geometries obtained according to

Fig. 5. Experimental and fitted binding curves constructed for the evaluation of the binding constant $K$ by monitoring resonance arising from heme 8-methyl of cyt c. (A) For wild-type ($K = 2.2 \times 10^4 \pm 2.3 \times 10^3$ M$^{-1}$), (B) the (E44/48/56A/D60A) mutant ($K = 5.1 \times 10^3 \pm 1.1 \times 10^3$ M$^{-1}$), respectively. Cyt c solutions (1 mM) were prepared in 1 mM phosphate buffer (pH 7.0) while the concentration of cyt b5 was varied from 0 to 2.5 mM.

Fig. 6. Dependence of the value of $K$ for the cyt b5±cyt c complex on ionic strength. (●) Values obtained by Muak et al. [52] (▲) Rodriguez-Marahón et al. [51] and (■) Value of $K$ from this work.

Fig. 7. 500 MHz 1D NMR paramagnetic difference spectra of: (A) 4 mM ferricytochrome c + 34 mol of [Cr(oxalate)3]$^{3+}$ per 100 mol of cyt c at pH 7.2; (B) 4 mM ferricytochrome c, 4 mM ferricytochrome b5 mutant + 34 mol of [Cr(oxalate)3]$^{3+}$ per 100 mol of cyt c at pH 7.2; and (C) 2 mM ferricytochrome b5 mutant +95 mol of [Cr(oxalate)3]$^{3+}$ per 100 mol of cyt b5 at pH 6.8. The resonances of Ile85, Thr28, Ile81, Thr89, Ala83, Met65, Thr19, Phe82 and His26 are marked. All spectra were recorded in D$_2$O at 298 K. The symbol (*) represents the resonances of ferricytochrome b5 mutant.

Lys13, Lys27, Lys72 and Lys79 of cyt c and the carboxyl group of Glu48, Glu44, Asp60 and the exposed heme propionate of cyt b5, respectively. Based on Brownian dynamics simulations in conjunction with kinetic measurements, Northrup et al. [10] proposed that the most frequently encountered docking geometries involved hydrogen bonding interactions of the following residues (cyt b5±cyt c): Glu48–Lys13, Glu56–Lys87, Asp60–Lys86, and heme–Tml72 with an average electrostatic energy of $-13.0$ kcal-mol$^{-1}$. The electrostatic stabilization energy of another less stable mode of interaction, which was identical with what was proposed by Salemme, was $-6.4$ kcal-mol$^{-1}$. When Glu44, Glu48, Glu56 and Asp60 were mutated into alanine, it could be predicted that the salt bridges formed by these residues would break. Thus, the most favorable bonding geometries obtained according to
the above two proposed models would not occur or would be disrupted to a great extent. However, our studies show that the binding and the ET between the mutant and cyt c did occur. Moore et al. [7] indicated that at least six lysine residues of cyt c were involved in the binding with cyt b5 by 2D $^{13}$C/$^1$H NMR studies. Our competition results indicate that it was highly possible that there were other protein association geometries, in which besides heme 6COO$^-$, other negatively charged residues around the heme exposed edge of cyt b5 mutant may have interacted with site III of cyt c. Probably these association geometries also occurred between wild-type cyt b5–cyt c. As far as the obtained association constant was concerned, they were relatively less stable than the above two proposed hypothetical models.

In fact, the X-ray crystallographic studies [43] revealed that 10 carboxyl residues outlined heme of bovine cyt b5 played a role in the interaction with other proteins. Mutation of Glu60, which is thought to bind to Lys72 of site III, to alanine might hand over its role in the interaction with cyt c to its neighbor of the same residue type, Glu59. Based on the same consideration, Glu44 might transfer its interaction to its neighbor Glu43, the latter is perhaps involved in the recognition process for the interaction of cyt b5 with NADH–cyt b5 reductase [53]. In summary, it is most likely that the interface of these two proteins is slightly shifted away from the hypothesized heme exposed domains suggested by Salemme [9] and Northrup et al. [10].

Concluding remarks

We have reported a high-quality structure of oxidized bovine liver microsomal cyt b5 mutant (E44/48/56A/D60A) and studied the binding and recognition between the mutant and cyt c, which provide a further insight into the interaction between cyt b5 and cyt c. The mutation of the four negatively charged residues to alanine did not bring about significant changes in the whole folding and secondary structure. The smaller association constant between the mutant and cyt c indicated that electrostatic interactions played an important stabilizing factor in the association of the cyt b5–cyt c complex. The results of competition between ferricytochrome b5 mutant and [Cr(oxalate)$_2$]$^{3+}$ for ferricytochrome c show that besides the two proposed hypothetical models, other association geometries probably also occur in the interaction between wild-type cyt b5 and cyt c.

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REFERENCES


**SUPPLEMENTARY MATERIAL**

The following material is available from http://www.blackwell-science.com/ejb/

Table S1. Chemical shifts (p.p.m.) of assigned diamagnetic proton resonances of cytochrome *b*5 mutant (E44/48/56A/D60A).
Table S2. Chemical shifts of assigned heme proton resonances of cytochrome b5 mutant at 303 K and pH 7.0. Resonance assignments are obtained at 303 K in 10 mM phosphate, pH 7.0.

Table S3. Distance constraints (Å) used in solution structure calculations.

Table S4. Pseudocontact shift constraints used for structure calculations.

Fig. S1. Schematic representation of the five stranded β-sheet of cyt b5 mutant.