SHORT COMMUNICATION

The influence of Glu44 and Glu56 of cytochrome $b_5$ on the protein structure and interaction with cytochrome $c$

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The gene encoding trypsin-solubilized bovine liver microsomal cytochrome $b_5$ (82 residues in length) has been mutated, in which the codons of Glu44 and Glu56 were changed to those of Ala. The mutated genes were expressed in Escherichia coli successfully and three mutant proteins (E44A, E56A and E44A/E56A) were obtained. The UV-visible, CD and 1H NMR spectra of proteins have been studied. The results show that the mutagenesis at surface residues does not alter the secondary and tertiary structures of cytochrome $b_5$ significantly. The interactions between recombinant cytochrome $b_5$ and its mutants with cytochrome $c$ were studied by using optical difference spectra. The results demonstrated that both Glu44 and Glu56 of cytochrome $b_5$ participate in the formation of a complex between cytochrome $b_5$ and cytochrome $c$.

Keywords: cytochrome $b_5$–$c$ complex/mutagenesis/protein structure

Introduction

Cytochrome $b_5$ is a membrane-bound protein in microsomes. The microsomal protein consists of a water-soluble, heme-binding domain (mol. wt ~10 kDa) and a short, hydrophobic tail that serves to anchor it to the endoplasmic reticulum membrane (Spatz and Strittmatter, 1971). This heme-containing protein is involved in a variety of electron transfer processes in biological systems, such as fatty acid desaturation (Strittmatter et al., 1974), methemoglobin reduction (Hultquist et al., 1984) and the cytochrome P-450 catalytic cycle (Cohen and Estabrook, 1971).

Based upon the computer simulation of three-dimensional structures of cytochrome $c$–cytochrome $b_5$, an electron transfer model of exposed heme edge–edge has been proposed by Salemme and co-workers (Salemme, 1976; Wendoloski et al., 1987). Several experimental studies have indicated a functional role for some of these negatively charged side chains in the formation of a protein complex between cytochrome $b_5$ and positively charged reaction partners (Ng et al., 1977; Bonfils et al., 1981; Poulos and Mauk, 1983; Livingston et al., 1985; Rodgers et al., 1988; Willie et al., 1993). For the cytochrome $b_5$–cytochrome $c$ protein complex, recognition will be provided by the electrostatic interaction of charged surface residues between the two proteins. The Brownian dynamic simulations (Northrup et al., 1993) predicted that there are two predominant classes of interaction in the cytochrome $b_5$ and cytochrome $c$ complex. The most predominant interaction involves the quartet of Glu48–Arg13, Glu56–Lys87, Asp60–Lys86 and heme propionate–Tml72 (cytochrome $b_5$ residues listed first). The second most important complex was Salemme's model with interactions of Glu44–Lys27, Glu48–Arg13, Asp60–Tml72 and heme propionate–Lys79. Obviously, there is a debate whether Glu44 or Glu56 is involved in the formation of the cytochrome $b_5$–cytochrome $c$ complex.

In an attempt to study the influence of the acidic acids surrounding the heme ring on the protein structure and the interaction with cytochrome $c$, we mutated the gene that encodes trypsin-solubilized bovine microsomal cytochrome $b_5$, in which Glu44 or/and Glu56 were changed to Ala44 or/and Ala56. (The number is according to the Mathew et al.'s result (1971)). The mutated genes were expressed at a high level in Escherichia coli. In addition, the purified proteins were characterized by amino acid composition analyses and electrospray mass spectrometry. The structural study of the proteins was performed by UV-visible, CD and 1H NMR techniques. The interaction between cytochrome $b_5$ and cytochrome $c$ was studied by optical difference spectra.

Materials and methods

Materials

T4 DNA polymerase, ligase, polynucleotide kinase and restriction enzymes EcoRI and HindIII were purchased from Biolabs. Ribonuclease A, X-gal and IPTG were obtained from Sigma. [α-32P]dATP and [γ-32P]dATP were purchased from Amer sham. Hen egg white lysozyme and deoxribonuclease I were obtained from Sino-American Biotechnology Co. All other chemicals were of reagent grade.

Mutagenesis, expression and purification of cytochrome $b_5$

The single-point mutations were accomplished by the method of Zoller and Smith (1983) and the double-point mutation was performed using the Kunkel (1985) method. The codon of Glu (GAA) of trypsin-solubilized cytochrome $b_5$ was mutated to that of Ala (GCT). The mutated genes were ligated into EcoRI–HindIII-cut pUC19 from M13 RF DNA and transformed into E.coli JM83. The DNA sequences in both the single and double strands were determined using dideoxy chain termination methods (Sanger et al., 1977). The expression of genes and protein purification were performed according to Funk's method (Funk et al., 1990).

Analysis of amino acid compositions

The purified protein was sealed in evacuated tubes with 6 M HCl and hydrolyzed at 110°C for 20 h. The amino acid standard and the hydrolyzed samples were reacted with ninhydrin. The reactants were detected at 570 nm in a Hitachi 835-50 Analyzer.

Electrospray mass spectrometry

Electrospray mass spectrometry was performed on a Quattro MS/MS system (VG Co., UK) equipped with an electrospray ionization system for mass spectrometry (Analytic Co., USA).

UV-visible and CD spectral studies

The UV–visible spectra were acquired using a Hewlett-Packard 8452A diode array spectrophotometer. The CD spectra were
obtained on a Jasco-20C CD spectrometer (with DP-500N data processor). The ferrous forms of cytochrome \( b_5 \) were obtained by adding Na\(_2\)S\(_2\)O\(_4\) reductant to the ferric protein solutions.

**\(^{1}H\) NMR spectroscopy**

\(^{1}H\) NMR spectra were recorded on a Bruker MSL-300 spectrometer equipped with an Aspect 3000 computer at 20°C and referenced to 1,4-dioxane (with a resonance at 3.74 p.p.m.). All samples contained 4 mM protein in 20 mM phosphate (pH* 7.0). The pH* was directly measured using a Metrohm 654 pH meter equipped with a Russell combined pH electrode. The pH* values are not corrected for isotope effects.

**UV–visible difference spectral studies**

Water purified with a Vaponics purification system to a resistance of 17–18 MΩ was used in the preparation of all solutions. Phosphate buffers of desired pH and ionic strength were prepared from monobasic phosphate sodium salt and sodium hydroxide. Horse heart cytochrome \( c \) (type VI) was purchased from Sigma Chemical Co. and purified by CM-cellulose cation-exchange chromatography to remove deamidated and polymeric cytochrome (Brautigan et al., 1978). The purified protein was concentrated and exchanged into phosphate buffers and stored at \(-20°C\). The protein concentration was determined with values of \( \varepsilon_{409} = 106 \, 100 \, \text{M}^{-1} \, \text{cm}^{-1} \) and \( \varepsilon_{414} = 117 \, 000 \, \text{M}^{-1} \, \text{cm}^{-1} \) for ferricytochrome \( c \) and \( b_5 \) respectively. Difference spectra were obtained using a tandem mixing cell and determined on a Hewlett-Packard 8452A diode array spectrophotometer at 25°C.

**Results and discussion**

Cytochrome \( b_5 \) is a well-folded globular metalloprotein. Based on the protein complex model of cytochrome \( b_5 \) and cytochrome \( c \) (Mauk et al., 1986; Northrup et al., 1993), Glu44 and Glu56 were chosen to be mutated into Ala44, Ala56 or Ala44/56 to break the intermolecular salt bridges and hydrogen bonds, to minimize the structural alteration of cytochrome \( b_5 \) as predicted by theoretical calculations (Garnier et al., 1978).

Two synthesized oligonucleotides were used to mutate the gene of cytochrome \( b_5 \). The positive clones were selected by hybridization with the primers labeled with \(^{32}P\) and confirmed by DNA sequencing. The mutated genes were cloned to the expression vector pUC19 and transformed into JM83. In the experiment, the high expression could be achieved when bacteria were incubated at a low level of aeration and a long duration of incubation. Yields of \(-12\) mg of purified proteins per liter of culture were obtained. The amino acid composition analysis showed that the Ala:Glu ratios in cytochrome \( b_5 \) wild type, E44A, E56A and E44/56A are 4:13, 5:12, 5:12 and 6:11 respectively, which is consistent with the gene sequence compositions. Electrospray mass spectrometry of wild type and E44A, E56A and E44/56A mutant cytochrome \( b_5 \) indicated that the molecular weights of the proteins are 9461.5 ± 1.3, 9403.4 ± 1.3, 9403.4 ± 1.3 and 9345.5 ± 0.6 Da respectively. These values correspond to the molecular weights calculated from the amino acid compositions of the apocytochrome without the N-terminal methionine residue (9461.3, 9403.3, 9403.3 and 9345.3 Da respectively). These results confirm the completely successful mutagenesis.

With regard to the structural comparisons between the recombinant wild type cytochrome \( b_5 \) and the mutant proteins, the CD spectra of cytochrome \( b_5 \) and the mutants were studied in the region of 195–580 nm. The CD spectra of the mutated proteins are similar to that of the wild type protein, which indicates that the secondary structures of the proteins are the same and that the micro-environment of the heme is not disturbed significantly, in accordance with the results of UV–visible spectra of the proteins. This is expected since in the designing of a mutants the minor structural alteration of the proteins was claimed in order to study electrostatic effects on the electron transfer reactions of cytochrome \( c/b_5 \).

Detailed conformational information can be obtained from NMR study. The \(^{1}H\) NMR spectra of all cytochrome \( b_5 \) proteins were studied in a wide chemical shift region. The upfield field spectra are shown in Figure 1. The chemical shifts assigned to the heme and heme ligand resonances are listed in Table I. From these spectra, three points can be observed. First, these spectra are similar to those previously published for the tryptic-solubilized fragments of bovine liver microsomal cytochrome \( b_5 \) (Mclachlan et al., 1986, 1988) and indicate that the isolated proteins are in the low-spin ferric state. A preliminary comparison of the NMR spectra for individual mutant proteins provides evidence that only subtle structural alterations were detected. Comparatively, the changes in the proton resonances of pyrrole rings I and III are bigger than those of pyrrole rings II and IV. In particular the 1-methyl, 2-vinyl H\(_4\), 5-methyl, H\(_a\) and H\(_b\) of the 6-propionic group exhibit a chemical shift around their original positions more often. Second, unlike the \( c \)-type cytochrome proteins, the \( b \)-type cytochromes present in two conformationally heterogeneous forms. The so-called 'minor' component has the heme orientation with respect to the one characterized in the initial X-ray data. On the other hand, the 'major' component possesses a conformation so that the orientation of heme in the minor component is rotated 180° about the \( \alpha, \gamma \)-meso axis. This is also the situation which is clearly seen in the low field of the \(^{1}H\) NMR spectra of the recombinant and mutated cytochrome \( b_5 \) proteins. Many peaks of the minor isomer are present in the low field portion of
The optical difference spectra in the 320-500 nm region, which resulted from the interaction of cytochrome c with recombinant wild type cytochrome b₅, E44A, E56A and E44/56A at pHs 7.0 and 7.75, are shown in Figure 2. The intensity of the difference is a measure of the formation of protein complex as indicated by previous literature (Mauk et al., 1982). The maximum differences of absorbance produced on mixing these proteins occur at 418 nm for wild type cytochrome b₅ and E56A, at 420 nm for E44A and at 422 nm for E44/56A at pH 7.0. The maximum differences of absorbance at pH 7.75 are the same as those at pH 7.0 except at 420 nm for E44/56A.

As we mentioned above there are several binding models between cytochrome b₅ and cytochrome c. The high pressure technique study together with site-directed mutagenesis showed that all four carboxyl groups of rat liver cytochrome b₅ described in Salemme’s model are involved in salt bridges with cytochrome c, but Glu13, Glu37, Glu56 and Asp66 are not (Rodgers and Sligar, 1991). However, the difference spectrum study indicated that the heme propionate of cytochrome b₅ participates in the association with cytochrome c although the blockage of cytochrome b₅ heme 6-propionate does not result in the simple loss of complex affinity with cytochrome c. Here, difference spectra studies on the cytochrome b₅ mutants (E44A, E56A, and E44/56A) with cytochrome c were carried out to examine the role these negatively charged groups play in the formation of the protein complex. At pHs 7.0 and 7.75, the maximum differences of absorbance produced when mixing cytochrome c with wild type cytochrome b₅ or the mutants is in the following order: wild type > E56A > E44A > E44/56A. At the same time, we also found the maximum difference of absorbance increased with an increase in the pH value as described previously (Mauk et al., 1986). Our result convincingly demonstrates that not only is Glu44 involved in the interaction between cytochrome b₅ and cytochrome c, but that Glu56 also participates in the association of these two proteins. There are more than four carboxyl groups of cytochrome b₅ contributing to the interaction with cytochrome c. It also makes us believe that cytochrome b₅ and cytochrome c can form a flexible association complex which depends significantly on the solution conditions such as the pH and the ionic strength.

In conclusion, this study has demonstrated that site-directed mutagenesis at surface residues of cytochrome b₅ did not produce significant changes in cytochrome b₅’s gross structure. Moreover, our study of the difference spectra demonstrates that in the formation of the cytochrome b₅-cytochrome c spectrums and this is evidence of the existence of two heterogeneous conformational species in solution. The resonances at 31.7 and 22.2 p.p.m. are assigned to the 3'-methyl of the minor orientation and 5-methyl of the major orientation respectively. Integration of these peaks provides the ratio for the interconversion of these two conformers. A 1:6.4 ± 0.5 ratio of the minor species to the major species was obtained with the recombinant and mutant proteins instead of a ratio of 8.9 which was reported in the case of bovine liver microsomal cytochrome b₅ (McLachlan et al., 1986). The dispersal was interpreted by the dynamic existence of heme in the crevice (Lederer, 1994). Third, in the upfield spectra there is almost no difference observed between the spectra of the wild type and E56A; however E44A shows an identical spectrum to E44/56A. E44A only showed a slightly different spectrum from that of the wild type recombinant protein at the following resonances: β-Htrans and β-Hiso of heme 2-vinyl and Pro40 β-CH, α-meso H, 6-β CH and 7-α CH of heme propionate. E44/56A shows an identical spectrum to the E44A spectrum.

The optical difference spectra in the 320-500 nm region, which resulted from the interaction of cytochrome c with recombinant wild type cytochrome b₅, E44A, E56A and E44/56A at pHs 7.0 and 7.75, are shown in Figure 2. The intensity of the difference is a measure of the formation of protein complex as indicated by previous literature (Mauk et al., 1982). The maximum differences of absorbance produced on mixing these proteins occur at 418 nm for wild type cytochrome b₅ and E56A, at 420 nm for E44A and at 422 nm for E44/56A at pH 7.0. The maximum differences of absorbance at pH 7.75 are the same as those at pH 7.0 except at 420 nm for E44/56A.

Table I. Chemical shifts for the heme and heme ligand resonances of the cytochrome b₅ wild type and mutants

<table>
<thead>
<tr>
<th>Resonance</th>
<th>Chemical shift (δ p.p.m.)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>3-Methyl (minor)</td>
<td>31.74</td>
</tr>
<tr>
<td>8-Methyl (minor)</td>
<td>28.08</td>
</tr>
<tr>
<td>2a-Vinyl</td>
<td>27.96</td>
</tr>
<tr>
<td>5-Methyl</td>
<td>22.21</td>
</tr>
<tr>
<td>7-α-CH</td>
<td>19.02</td>
</tr>
<tr>
<td>His39 β-CH</td>
<td>16.90</td>
</tr>
<tr>
<td>6-α-CH</td>
<td>16.07</td>
</tr>
<tr>
<td>6-α-CH</td>
<td>15.74</td>
</tr>
<tr>
<td>3-Methyl</td>
<td>14.13</td>
</tr>
<tr>
<td>1-Methyl</td>
<td>11.91</td>
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</table>

All spectra were recorded at 20°C and in 20 mM phosphate, pH 7.0.
complex both Glu44 and Glu56 are involved. This result combines with the observation that a redox switch between reduced and oxidized cytochrome $b_5$ generates no noticeable conformational change in solution (Veitch et al., 1990) and leads us to believe that the hydrophobic core of cytochrome $b_5$ is constructed by a heme center tightly folded by the polypeptide chain. Comparatively speaking, it seems to us that the more compact hydrophobic core of cytochrome $b_5$ makes this protein more stable and less easily disturbed than the cytochrome $c$ protein. This is also consistent with our recent study showing that the midpoint redox potentials of these cytochrome $b_5$ mutants have rather similar values. Mutants exhibit unchanged redox potentials that can often be considered to lack the perturbation of the heme environment and the redox potential is a sensitive probe of structural perturbations, as stated by Caffrey and Cusanovich (1994). From the detailed knowledge of the structure of cytochrome $b_5$ mutants in the current study, we expect to characterize the electron transfer reactions of these proteins with the redox partner proteins.

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References