Importance of a conserved phenylalanine-35 of cytochrome $b_5$ to the protein’s stability and redox potential

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Phenylalanine-35, which is a residue of the hydrophobic patch on the surface of cytochrome $b_5$, has been mutated into Tyr35, His35 and Leu35 to elucidate the functions of the Phe35 and give further insight into the roles of the hydrophobic patch and/or aromatic network. The effects of these mutations on the heme environment, denaturation towards heating and the denaturant urea, redox potential and stability of protein were studied. The relative stability of cytochrome $b_5$ and its mutants towards heating has the order Phe35Tyr > wild type > Phe35Leu > Phe35His in the oxidized state and wild type > Phe35Tyr > Phe35Leu > Phe35His in the reduced state. All the mutants exhibit decreased reduction potentials: Phe35Tyr -66 mV, Phe35His -51 mV and Phe35Leu -28 mV, which are more negative than that of the wild type. The order of redox potential reflects the relative stability in the oxidized and reduced states. A method of producing multiple mutants at a single site of a gene is also described for the first time.

Keywords: cytochrome $b_5$ / mutagenesis / protein stability / redox potential

Introduction

Cytochrome $b_5$ is a membrane-bound protein, but it can be released by proteolysis to yield a soluble domain that contains a non-covalently bound heme group. This hydrophilic domain is involved in electron transfer with a variety of proteins. Most of the studied interactions of cytochrome $b_5$ with other proteins indicated that a cluster of negatively charged residues surrounding the heme group on cytochrome $b_5$ were involved. The carboxyl-containing residues on cytochrome $b_5$ are used for binding to the basic residues on the protein partners, such as cytochrome $c$ (Mauk et al., 1995), methemoglobin (Poulos and Mauk, 1983), cytochrome P-450 (Stayton et al., 1989) and metmyoglobin (Livingston et al., 1985).

On the surface of cytochrome $b_5$, there is a hydrophobic patch of 350 Å² that is surrounded by negatively charged residues (Mathews and Czerwinski, 1976). It represents an exception to the rule that hydrophobic residues are grouped primarily in the interior of a protein molecule. The patch consists of the hydrophobic groups, Phe35, Pro40, Leu70 and Phe74. Interestingly, this patch is totally conserved among different species with the exception of residue 74, which has a conservative substituent of tyrosine. Furthermore, the residues Phe35 and Phe/Tyr74 also form an aromatic network with His39, which is one of the axial ligands of heme b. Phe/Tyr74 is partially exposed on the surface of cytochrome $b_5$ and its buried surface contacts the heme crevice (Lederer, 1994). It has been claimed that the hydrophobic patch (and/or aromatic network) could be the second electron transfer site of cytochrome $b_5$ with its amphipathic redox partners such as cytochrome p-450 and cytochrome p-450 reductase (Vergeres et al., 1993). Studies of Tyr74Lys mutant of the rat membrane-bound cytochrome $b_5$ demonstrated that Tyr74 is not necessary for the binding and electron transfer function of cytochrome $b_5$. It is also reported that this residue is not a determinant of the reduction potential or spectroscopic properties of the protein, but rather shields the heme from the solvent, so enhancing heme’s association with the cytochrome $b_5$ polypeptide chain (Vergeres et al., 1993).

Compared with Tyr/Phe74, Phe35 is closer to the heme pocket and is an invariant residue in eight species of cytochrome $b_5$ (Mathews, 1985). In the family of $b_5$-like cytochromes, Phe35 is a more highly conserved residue with no other substitutions than tyrosine (Lederer, 1994). To illustrate the functions of Phe35 and gain further insight into the roles of the hydrophobic patch and/or aromatic network, three cytochrome $b_5$ mutants, Phe35Tyr, Phe35His and Phe35Leu, were studied in this work. The Phe35Tyr mutation was chosen because it is a conservative substitution in the family of $b_5$-like cytochromes (Lederer, 1994). Further, compared with Phe35, in Tyr35 and His35 mutants a slightly hydrophilic group, tyrosine or histidine, was introduced. On the other hand, in Leu35 mutant the leucine is still a hydrophobic residue, but with a smaller size, and breaks the aromatic network. The effects of these mutations on the heme environment, denaturation towards heat and the denaturant urea, redox potential and relative protein stability were studied. The results demonstrate that Phe35 plays an important role in the stability of the protein, and mutation at this site shifts the redox potentials considerably. This phenomenon is usually seen in substitution of interior residues of cytochrome $b_5$. The relative stability of the mutants towards heating has the order Phe35Tyr > wild type > Phe35Leu > Phe35His in the oxidized state and wild type > Phe35Tyr > Phe35Leu > Phe35His in the reduced state. All the cytochrome $b_5$ mutants exhibit decreased redox potentials with the largest difference of 66 mV between Phe35Tyr and the wild type. The order of redox potentials is Phe35Tyr < Phe35His < Phe35Leu < wild type, which reflects the relative stability in the oxidized and reduced states. In this paper a method of producing multiple mutants at a single site of a gene is also described for the first time.

Materials and methods

DNA restriction endonucleases, T4 DNA polymerase, ligase and kinase were purchased from Biolabs. [γ-32P]ATP was from Amersham. The pUC19 plasmid containing the synthetic gene encoding the trypsin-solubilized bovine liver microsomal cytochrome $b_5$ (82 residues in length) was a generous gift from Professor A.G.Mauk (Funk et al., 1990). The other
bio-products were from Sino-American Biotechnology. All chemicals were of reagent grade.

**Mutagenesis, expression and purification of cytochrome b**

Unless specified otherwise, all DNA manipulations were performed as described by Sambrook et al. (1989). Site-directed mutagenesis of the gene coding for the trypsin-solubilized cytochrome b was accomplished using phage M13mp19 as described by Sambrook et al. (1989). Three partially degenerate oligonucleotides:

\[
\begin{align*}
5^\prime-\text{CTGACTAAATCTGGAAGAGC-3}^\prime & \quad \text{Phe35 (wild type)} \\
\text{TAT} & \quad \text{Phe35Tyr} \\
\text{CAT} & \quad \text{Phe35His} \\
\text{CTG} & \quad \text{Phe35Leu}
\end{align*}
\]

were synthesized in an Applied Biosystems 381A DNA synthesizer. The synthesis of the oligonucleotides was started from the first base at the 3'-end to the tenth base; then the oligonucleotide (10 bases long) was divided into three fractions. Each fraction of oligonucleotide was separately extended according to one of the three brackets indicated above. Then the three fractions were combined and subsequently synthesis was continued from the fourteenth base at the 3'-end and completed at the 5'-end. The oligonucleotide mixture was purified by polyacrylamide gel electrophoresis under non-denaturing conditions, followed by reversed-phase liquid chromatography with a C18 cartridge. The 5'-end of the oligonucleotides was phosphorylated with T4 polynucleotide kinase and ATP. The oligonucleotide primer mixture (0.1 pmol) was hybridized with 0.1 pmol of recombinant M13 single-stranded DNA template containing uridine and then it was extended with T4 DNA polymerase (12U) to create a double-stranded DNA. Nicks in the duplex were sealed with T4 DNA ligase (6U). After this, the duplex DNA was transformed into the *Escherichia coli* host TG1. The single-stranded mutant DNA (0.5 ml, 500 ng) from each isolated plaque was precipitated using 0.1 ml of 20% PEG 8000, 2.5 M NaCl. The precipitate was collected by centrifugation and then dissolved in 20 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The concentrated single-stranded DNA (5 µl, 100 ng) for each of mutant candidates was separately spotted on a dry sheet of nitrocellulose (10x5 cm), using single-stranded M13mp19/wild type cytochrome b5 DNA as controls (Yang et al., 1990). 32P-labeled oligonucleotide mixture used for mutation was poured into the hybridization solution and kept at 22°C for 10 h. The nitrocellulose filter was then washed with 6×SSC (0.9 M NaCl, 0.09 M Tris–sodium citrate, pH 7.0) at 42, 62, 66, 68 and 70°C. Autoradiography was carried out after each washing. The mutated cytochrome b5 genes were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) and then ligated into *E. coli* host JM83. The expression of genes and the purification of protein were performed according to Mauk’s method (Funk et al., 1990).

**Electrospray mass spectrometry**

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

**Spectroelectrochemical measurements**

Spectroelectrochemical measurements were performed with a Model 8452A diode-array spectrophotometer (Hewlett-Packard, USA) at room temperature. The cell was constructed as described previously (Baymann et al., 1991). The working electrode was a gold minigrid optically transparent thin-layer electrode (OTTLE, 400 wires per inch) with quartz windows on each side. The counter electrode was a platinum wire immersed in the protein solution. A saturated calomel electrode (SCE) was used as the reference electrode. Ru(NH₃)₆Cl₃ was added as a mediator of the protein solution. Spectra of fully reduced protein and fully oxidized protein were recorded at −350 and +120 mV (versus SCE), respectively. The spectra were recorded at about every 20 mV started from −350 mV (versus SCE). The wild type and mutants of cytochrome b5 were dissolved in phosphate buffer, pH 7.0, with a protein concentration of ~3 µM. Each sample was then carried out at a temperature range from 25 to 90°C and for each experiment a period of 20 min was left between measurements to ensure that the sample had reached equilibrium. The temperature was determined directly in the cuvette holder and was maintained to within ±0.2°C. Reduction of cytochrome b5 was performed according to Newbold et al. (1992). For denaturation by urea, wild type and mutant cytochrome b5 were dissolved in 100 mM sodium phosphate buffer, pH 7.0, at a final protein concentration of ~4 µM. Each sample contained different concentrations of urea, ranging from 0 to 9.5 M in 0.5 M increments. The proteins were allowed to equilibrate with urea solution for 18 h at 30°C until the absorbance at 412 nm had stabilized and spectra were recorded from 200 to 800 nm in a Model 8452A diode-array spectrophotometer (Hewlett-Packard).

**Results**

**Mutagenesis, expression and purification of cytochrome b**

The temperature of removal of individual 32P-labeled mutagenic oligonucleotides from mixtures by dot blot hybridization screening was estimated according to the equation

\[ T_D (°C) = 4 (°C) \times (G–C) \text{ pairs} + 2 (°C) \times (A–T) \text{ pairs} \]

where \( T_D \) is the temperature for 50% dissociation of perfectly matched hybrids. From calculations, \( T_D \) of wild type cytochrome b5 is 62°C and those of Phe35Tyr, Phe35His and Phe35Leu are 66, 68 and 70°C, respectively. Figure 1A shows the results of autoradiography. After being washed at 42°C, the autoradiographic background on the film was minimized (Figure 1A). When the temperature reached 62°C, the M13mp19/wild type cytochrome b5 (first and second blots) showed positive results, as did the other 12 non-mutant specimens, while 16 blots remained dark (Figure 1B). When the filter was washed at 66°C, another two dark blots disappeared and 14 blots remained dark (Figure 1C). After washing at 68°C, there were only 10 dark blots left (Figure 1D). On washing at 70°C, all the remaining 10 blots disappeared. From dot blot hybridization screening for mutants, the 28 mutant candidates were divided into four groups as expected:
Conserved Phe35 of cytochrome b5

Table I. Reduction potentials of bovine cytochrome b5 (sodium phosphate buffer, \( \mu = 0.1 \text{ M}, \text{pH 7.0}, \text{room temperature} \))

<table>
<thead>
<tr>
<th>Cytochrome b5</th>
<th>( E_0 ) (mV vs SHE)</th>
<th>Nernst slope (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2</td>
<td>58.3</td>
</tr>
<tr>
<td>Phe35Leu</td>
<td>-26</td>
<td>60.4</td>
</tr>
<tr>
<td>Phe35His</td>
<td>-49</td>
<td>59.9</td>
</tr>
<tr>
<td>Phe35Tyr</td>
<td>-64</td>
<td>59.5</td>
</tr>
</tbody>
</table>

of Phe35His per liter of culture. The purified proteins showed a single band on SDS–PAGE analysis (Sambrook et al., 1989). Electrospray mass spectrometry of wild type and Phe35Tyr, Phe35His and Phe35Leu mutants of cytochrome b5 indicated that the molecular weights of the protein are 9461.5 ± 1.3, 9478.1 ± 1.5, 9454.3 ± 0.4 and 9427.9 ± 2.3 Da, respectively. These values correspond to the molecular weights calculated from amino acid compositions of apo-cytochrome b5 (9461.3, 9477.3, 9451.3 and 9427.3 Da, respectively). These results confirm the completely successful mutagenesis.

Spectroelectrochemical studies

Since the redox potentials of heme proteins are influenced by the solvent exposure and local protein environment of the heme, comparison of the redox potentials of wild type and mutants is a sensitive assay for monitoring changes in the heme environment (Caffrey et al. 1991). Phe35Tyr, Phe35Leu and Phe35His proteins exhibit electrochemically reversible behavior as observed in the wild type cytochrome b5 by Funk et al. (1990), Reid and Mauk (1982) and Wang et al. (1997). The reduction potentials and Nernst slopes obtained for wild type and mutant proteins are shown in Table I. All the three mutants present a decreased redox potential with the order Phe35Tyr, Phe35His, Phe35Leu, wild type. The largest difference in \( E_0 \) is 66 mV between Phe35Tyr and wild type protein. The observation that all the three mutants display decreased redox potentials suggests that the oxidized state of the mutants has been stabilized with respect to the reduced state.

Thermal stability studies

Protein denaturation is a useful measure for studying the effects of site-directed mutagenesis on protein stability (Alber, 1989). Electronic spectra between 200 and 700 nm of Phe35Tyr, Phe35Leu and Phe35His are indistinguishable from those of the well characterized tryptic fragment of cytochrome b5 in both the oxidized and reduced states under similar solution conditions. The maximum absorbance of the Soret band was observed at 412 nm for the oxidized state and 424 nm for the reduced state. In view of this observation, it is possible to assess the thermal denaturation of mutant cytochrome b5 using the Soret peaks at 412 and 424 nm to compare their stability with that of the wild type (Newbold et al., 1992; Hewson et al., 1993; Vergeres et al., 1993). The thermal denaturation curves were derived by plotting the absorbance of cytochrome b5 at 412 nm for the oxidized state and at 424 nm for the reduced state as a function of temperature (Figure 2). The profiles of the denaturation curves indicate that cytochrome b5 unfolding is a single, cooperative transition between folded (F) and unfolded (U) states (Newbold et al., 1992; Hewson et al., 1993). The apparent transition temperatures \( T_m \) between the folded and unfolded states were estimated from the

Fig. 1. Results of autoradiography of dot blot hybridization screening for mutants of cytochrome b5. (A) After being washed at 42°C with 6x5 SSC; (B) after being washed at 62°C; (C) after being washed at 66°C; (D) after being washed at 68°C.
Fig. 2. Denaturation curves for wild type and mutant cytochrome b5. Fractional changes in (A) absorbance at 412 nm with increasing temperature for oxidized proteins, (B) absorbance at 424 nm with increasing temperature for reduced proteins and (C) absorbance at 412 nm with increasing urea concentration for oxidized proteins at 30°C. The data sets were constructed by assuming a two-state unfolding process and are shown normalized to facilitate comparison between each protein. Symbols correspond to Phe35His (●), Phe35Leu (▽), Phe35Tyr (□) and wild type (○). Buffer, sodium phosphate, (0.1 M), pH 7.0.

denaturation curves in Figure 2A and B. The equilibrium constants can be obtained from the equation:

\[ K_U = \frac{[A_{f}(T) - A_{U}(T)]/[A(T) - A_{U}(T)]}{[A(T) - A_{U}(T)]} \]  

(2)

where \( A_{f}(T) \) and \( A_{U}(T) \) are the absorbances of the folded and unfolded states of the protein at temperature \( T \), respectively and \( A(T) \) is the absorbance measured at this temperature. The free energy of unfolding can be calculated according to

\[ \Delta G_U = -RT \ln K_U \]  

(3)

A plot of \( \ln K_U \) against \( 1/T \) (or of \( \Delta G_U \) against \( T \)) allows an estimate of the enthalpy \( \Delta H_m \) and entropy \( \Delta S_m \) from the linear region around the transition temperature \( T_m \). These values are summarized in Table II. At the transition temperature, \( \Delta G_{U} = 0 \) and thus \( \Delta S_m = \Delta H_m/T_m \). The values of \( \Delta (\Delta G)_U \) were estimated from the relationship \( \Delta (\Delta G)_U = \langle \Delta S_m \rangle [T_m(\text{mutant}) - T_m(\text{WT})] \) (Caffrey et al., 1991; Newbold et al., 1992; Vergeres et al., 1993). \( \langle \Delta S_m \rangle \) is the average of the \( \Delta S_m \) values for wild type and mutant proteins (Vergeres et al., 1993). The free energy of denaturation at room temperature was estimated by linear extrapolation from the transition region to non-denaturing conditions, assuming a linear dependence of \( \Delta G \) on denaturing temperature. However, it is subject to errors (Pace, 1986). Therefore, the \( \Delta (\Delta G)_U \) values for the mutants are more accurately used to compare their stability relative to the wild type cytochrome b5 in the same oxidation state (Caffrey et al., 1991).
more affected. For the Phe35His mutant, both redox states are proteins obtained from the linear region around the transition existence of the Phe35His mutated gene, possibly owing to, Δ2 and 3. The difference in denaturation: Phe35Leu more stable to denaturation than the ferricytochrome b2

Table III. Thermodynamic data for thermal denaturation of cytochrome b5 (0.1 M sodium phosphate buffer, pH 7.0, 30°C)

<table>
<thead>
<tr>
<th>Cytochrome b5</th>
<th>Tm (°C)</th>
<th>ΔTm (°C)</th>
<th>ΔHm (kJ/mol)</th>
<th>ΔSm (J/mol.K)</th>
<th>Δ(ΔG)m (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized WT</td>
<td>66.2</td>
<td>–</td>
<td>298</td>
<td>878</td>
<td>–</td>
</tr>
<tr>
<td>Oxidized Phe35Tyr</td>
<td>69.6</td>
<td>3.4</td>
<td>361</td>
<td>1053</td>
<td>3.3</td>
</tr>
<tr>
<td>Oxidized Phe35Leu</td>
<td>59.1</td>
<td>–7.1</td>
<td>434</td>
<td>1306</td>
<td>–7.8</td>
</tr>
<tr>
<td>Oxidized Phe35His</td>
<td>54.9</td>
<td>–11.3</td>
<td>396</td>
<td>1207</td>
<td>–11.8</td>
</tr>
<tr>
<td>Reduced WT</td>
<td>76.7</td>
<td>–</td>
<td>345</td>
<td>986</td>
<td>–</td>
</tr>
<tr>
<td>Reduced Phe35Tyr</td>
<td>74.4</td>
<td>–2.3</td>
<td>358</td>
<td>1030</td>
<td>–2.3</td>
</tr>
<tr>
<td>Reduced Phe35Leu</td>
<td>68.8</td>
<td>–13.6</td>
<td>385</td>
<td>1120</td>
<td>–8.3</td>
</tr>
<tr>
<td>Reduced Phe35His</td>
<td>63.1</td>
<td>–13.6</td>
<td>492</td>
<td>1463</td>
<td>–16.7</td>
</tr>
</tbody>
</table>

Table III. Urea denaturation of bovine ferricytochrome b5 (0.1 M sodium phosphate buffer, pH 7.0, 30°C)

<table>
<thead>
<tr>
<th>Cytochrome b5</th>
<th>Cm (mol)</th>
<th>m (kJ/mol²)</th>
<th>Δ(ΔG)m (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7.1</td>
<td>–3.7</td>
<td>–</td>
</tr>
<tr>
<td>Phe35Tyr</td>
<td>8.3</td>
<td>–3.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Phe35Leu</td>
<td>4.9</td>
<td>–3.4</td>
<td>–7.8</td>
</tr>
</tbody>
</table>

Urea denaturation

Table III gives the results of urea denaturation studies of wild type, Phe35Tyr and Phe35Leu of ferricytochrome b5 and a decline in the intensity of the Soret peak with increase in urea concentration is shown in Figure 2C. These are similar to those seen in the thermal denaturation. The existence of one isosbestic point at 398 nm (not shown) also indicates a single cooperative unfolding process (Vergeres et al., 1993). Unfolding constants KU and ΔG_U were calculated by Equations 2 and 3. The difference in ΔG_U between the wild type and mutant proteins was also estimated at the midpoint of denaturation:

\[ \Delta(\Delta G)_U = \langle m D \rangle \Delta C_m (\text{mutant}) - C_m (\text{WT}) \]

Discussion

Oligonucleotide-directed mutagenesis is an important tool in genetic and protein engineering. By the method we developed, three mutants were obtained in a single process with a three partially degenerated oligonucleotide mixture. In the process of annealing, hybridization association rates are strongly dependent on salt concentration and temperature (Davis et al., 1994). Under our annealing conditions the hybridization association rates of three oligonucleotides encoding Phe35Tyr, Phe35His and Phe35Leu were different and dependent on their T_D values. The T_D order for three mutant oligonucleotides is Phe35Leu > Phe35His > Phe35Tyr. During the construction of the mutated genes, at the start a large excess of synthesized oligonucleotide mixture was used, but we only obtained the Phe35Tyr and Phe35Leu mutated genes and failed to find the existence of the Phe35His mutated gene, possibly owing to the competition among these oligonucleotides for ss-DNA. When the ratio of mixed oligonucleotides to ss-DNA was reduced to 1:1, the three mutated genes corresponding to Phe35Tyr, Phe35His and Phe35Leu were obtained. The efficiency of mutation was 10/28 for Phe35Leu, 4/28 for Phe35His and 2/28 for Phe35Tyr corresponding to the T_D order. In the process of the dot blot hybridization screening process, because of degeneracy of oligonucleotides, all the three oligonucleotides hybridized to all the blots which belong to four cytochrome b5 genes (wild type, Tyr35, Leu35, His35 ). As shown in Figure 1, after each washing, the intensity of autoradiography was decreased on the remaining blots. Concentrated ss-DNA was used for dot blot hybridization to make autoradiographic spots of the remaining positive hybridization blots visible after several washings.

The technique of site-directed mutagenesis is ideally suited for elucidating the functions of individual amino acid residues in protein. The improved method described in this paper is simple, efficient and universal for producing multiple mutants at a single site of gene, provided that the T_D values of the mutant oligonucleotides chosen are different. Instability of a protein may be the one of factors affecting the yield of the protein in the expression of the gene. In cytochrome c, a decrease in stability by 8.4 kJ/mol or more may significantly decrease the protein yield and in some cases...
it actually favors the unfolded state (Caffrey, 1994). The formation of a secondary structure of the RNA could be another main cause of low expression; however, nucleic acid and protein sequence analysis using the PC/gene software system (IntelliGenetics Inc., July 1, 1993) indicated that there is no such structure in the Phe35His and Phe35Leu genes, or in the wild type and Phe35Tyr genes of cytochrome $b_5$. Therefore, the low yield of cytochrome $b_5$ Phe35His mutant protein is attributable to the instability of the Phe35His protein. The instability of Phe35Leu also has some effect on the yield of the mutant protein.

The main components of protein stability that could be perturbed by mutation at interior groups include hydrophobic effect, van der Waals force, backbone conformation, hydrogen bonds and local polarity (Sandberg and Terwilliger, 1989). If the residue is located in the protein interior, the side chain's volume of the substituted residue is also an important factor that needs to be considered. Substitution of Phe35 by Leu and His decreases the side chain volume of 36 Å³ individually (Caffrey, 1994). When an interior residue is replaced by a smaller residue, the destabilization of protein results first from the smaller residue being generally less hydrophobic than the larger residue and thus the protein being destabilized. Second, the smaller residue creates an internal cavity in protein that results in a loss of stability by reduction of van der Waals interactions. The magnitude of these interaction has been estimated by mutagenesis studies of phage T4 lysozyme to be 20–30 cal/Å³ (Matthews, 1993). For Phe35Leu and Phe35His, the creation of a 36 Å³ cavity could cause destabilization of protein by ~3–4.5 kJ/mol.

The hydrophobic interaction is a dominant stabilizing force in proteins (Kauzmann, 1959) and an important driving force in protein folding (Dill, 1990). The relative hydrophobicity of the substitution residue must be considered because Phe35 closely contacts the heme group (Mathews and Czerwinski, 1976). The energetics of the hydrophobic effect have been estimated from studies of the partitioning of amino acids between a hydrophobic solvent such as octanol or ethanol and water (Fauchere and Plińska, 1983). According to these criteria, substitution of tyrosine, histidine and leucine for phenylalanine would generate 4.8, 13.0 and 0.4 kJ/mol of destabilizing energy, respectively, owing to the less hydrophobic nature of tyrosine, histidine and leucine (Fauchere and Plińska, 1983; Caffrey, 1994).

After hundreds and thousands years of evolution and natural selection, the wild type protein usually develops an optimal architecture to fulfil its biological functions. The artificial site-directed mutagenesis of proteins most frequently leads to a decrease in the stability of the proteins. Therefore, the increased stability observed here in the mutation of cytochrome $b_5$ is comparatively rare (Newbold et al., 1992; Caffrey et al., 1994). The explanation is that the mutation causes structural alterations of proteins. An example of studying the importance of hydrogen bonds to protein stability is given by the Tyr75Phe mutant of Rhodobacter capsulatus cytochrome $c_5$ (Caffrey et al., 1991). Substitution of the highly conserved Tyr75, which is also located proximal to the heme, by phenylalanine was shown to decrease the stability of the ferro state by 7.66 kJ/mol. This is an unexpected result on protein stability from a more hydrophobic substitution. Examination of the Rh.capsulatus cytochrome $c_5$ structure suggested that the decreased stability of the Tyr75Phe is due to the disruption of a hydrogen bond between the Tyr75 side chain and a highly conserved water molecule. Interestingly, the ferric state of Tyr75Phe exhibited a 1.46 kJ/mol increase in stability, suggesting that the Tyr75 is not part of a stabilizing hydrogen-bonding network in the oxidized state. In our study, the Phe35Tyr mutant of cytochrome $b_5$ is 3.3 kJ/mol more stable in the oxidized state and 2.3 kJ/mol less stable in the reduced state compared with the same redox state of wild type cytochrome $b_5$. Because tyrosine is a less hydrophobic residue than phenylalanine, this result is unexpected. As mentioned in the literature (Clothia, 1975), in a well structured protein more than 50% of the polar groups (i.e. NH, NH₂, C=O, OH) are involved in hydrogen bonds with other polar groups of the proteins. Moreover, the majority of the remaining polar groups of proteins are involved in hydrogen bonds with water molecules (Frey, 1993). It is very possible that there is another hydrogen bond formed in Phe35Tyr ferrocyanochrome $b_5$. Mutagenesis studies of phage T4 lysozyme have shown that a single hydrogen bond typically contributes 4–6 kJ/mol to protein stability (Alber et al., 1987), which coincides with the fact that the Phe35Tyr ferrocyanochrome $b_5$ is 3.3 kJ/mol more stable while ferrocyanochrome $b_5$ is 2.3 kJ/mol less stable than the wild type protein. However, that needs to be proved. Moreover, the redox potential of the Phe35Tyr mutant shifts 66 mV negatively compared with the wild type protein. It is understandable that stabilization of the oxidized state and destabilization of the reduced state will result in a decrease in the redox potential (Caffrey et al., 1991). In the case of the Phe35His mutant, the redox potential is 51 mV more negative with respect to the wild type, which means the ferric form of Phe35His is more stable than reduced Phe35His. The increase in stability in the ferric state could be due to the introduction of a partially positively charged histidine adjacent to the hydrophobic heme crevice.

It is also interesting to make a comparison on mutation at sites of Tyr74 and Phe35 of cytochrome $b_5$. When mutation occurred at Phe35, even though the hydrophobicity and polarity of the residues only showed a slight change, the reduction potentials of the protein shifted negatively by several tens of millivolts (as shown in Table I). Then, when a slightly hydrophilic residue, tyrosine at position 74 of the protein polypeptide chain, was mutated into a lysine, a +1 positively charged residue, the potential shifted by only a few millivolts. Probably the location of the residue and its steric arrangement account for their different performances. The Tyr74 is situated closer to the protein surface and the long aliphatic chain [–(CH₂)₄–] of lysine is partially buried in the protein and makes it easy for the terminal hydrophilic amine extending to the heme pocket. On contrary, the Phe35 is buried deeper in the interior of the protein, and when the hydrophobic residue of phenylalanine was substituted by a slightly hydrophilic hydroxyphenyl or imidazole, it appeared to seriously disturb the hydrophobicity of the heme pocket, leading to shifts of reduction potentials. At the same time, when the Phe35 was replaced by the another hydrophobic residue, leucine, about a 30 mV $E'_o$ shift was observed. This is more likely to be due to either the side chain of leucine extending to the heme pocket, disturbing the heme conformation, or the volume decreasing in the residue substitution (36 Å³), creating an interior cavity, which alters the dielectric properties of the electron transfer medium. The structural characterization and comparison of wild type and mutants at Phe35 of cytochrome $b_5$ are currently being studied by two-
dimensional NMR techniques to elucidate the mechanism of the destabilization of the reduced state and stabilization of the oxidized state.

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