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The effect of mutation at valine-45 on the stability and redox potentials of trypsin-cleaved cytochrome b_5☆

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Abstract

In an attempt to elucidate the determinants of redox potential and protein stability in cytochrome b_5, three mutants at a highly conserved residue Val45, which is a member of heme hydrophobic pocket residues have been characterized. The V45Y mutant was designed to introduce a bulkier residue and a hydroxyl group to the heme pocket. The mutants V45H and V45E were constructed to test the effect of positive and negative charge on the stability and redox potential of proteins. The influence of these mutants on the protein stability towards thermal, urea, acid, ethanol and on the redox potential were studied. It is concluded that the decrease of hydrophobic free energy and the larger volume of the tyrosine make the phenylhydroxyl group of tyrosine still sitting inside the hydrophobic pocket, while the side chain of the mutant V45E and V45H shift away from the heme pocket. The redox potentials of mutants V45Y, V45H, V45E and wild-type of cytochrome b_5 are -35 mV, 8 mV, -26 mV and -3 mV, respectively. The bigger change of the V45Y on redox potential is due to the close contact between the hydroxyl group and the heme, while the changes of the V45E and V45H result from the alteration of charge density and distribution around the heme. Different relative stability of these mutants towards heat have been observed with the order: WT > V45Y ~ V45H > V45E being both in the oxidized and reduced state. The relative stability induced by addition of urea decreases in the order: WT > V45Y > V45H > V45E. These results suggest that the difference in the hydrophobic free energy is a major factor contributing to the stability of the Val45 mutants. Also the loose of the helix III in the mutant V45E makes it more unstable. These results indicate that residue Val45 plays an important role in the stability and redox potential of the protein.

Keywords: Cytochrome b_5; Mutagenesis; Redox potential; Protein stability

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1. Introduction

Cytochrome b_5 (Cyt b_5) is a membrane-bound protein and can be proteolysed to yield a soluble domain which contains a non-covalently bound heme group. This hydrophilic domain is involved in electron transfer with a variety of proteins, such as cytochrome c (Cyt c) [1–3], methemoglobin [4], cytochrome P450 [5,6] and metmyoglobin [7]. Of all these reactions the interaction between Cyt b_5 and Cyt c was thoroughly studied. Based on the computer simulation of three-dimensional structure of Cyt b_5 and Cyt c, several models are proposed which mainly concentrate on the electrostatic interaction between the cluster of negatively charged carboxyl residues surrounding the heme group of Cyt b_5 and positively charged residues of Cyt c [8,9].

Recently, more and more studies have been performed by site-directed mutagenesis on changing the residues, especially the axial ligation residues and negatively charged residues on the surface of cytochrome b_5 to illustrate their particular roles in modulating the stability and functions of the protein [1,2,4,8,10]. However, hydrophobic residues around the heme are relatively less investigated. Val45 of Cyt b_5 is a highly conserved residue in the heme hydrophobic pocket. It is located at the right side of the pocket and to the left of axial ligation residue His39 (Fig. 1), where it is directly in contact with the heme [11]. No doubt, this residue should play an important role on the property of Cyt b_5. Furthermore, Brownian dynamics simulations suggested that Val45 of Cyt b_5 may be involved in the hydrophobic interaction with Ala81 of Cyt c [9,12]. In an attempt to illustrate the structural and functional importance of Val45 residue, three mutants (V45Y, V45H and V45E) were constructed in this work. V45Y was designed to introduce a bulkier residue and a hydroxyl group. Residue Val45 was also substituted by a positively charged residue histidine (V45H) and a negatively charged residue glutamic acid (V45E). The present report examines the effect of Val45 mutations on the redox potential and on the stability of the proteins towards thermal, urea, acid and ethanol. The results show that residue Val45 plays an important role in the stability and redox potential of the protein. The different stability of the mutants towards heat have been observed with the order: WT > V45Y ~ V45H > V45E, being both in the oxidized and reduced state. The relative stability induced by urea decreases in the order: WT > V45Y > V45H > V45E. All three mutants exhibit similar stability towards acid and ethanol denaturation. The redox potentials of mutant V45Y, V45H, V45E and wild-type cytochrome b_5 are: −35 mV, 8 mV, −26 mV and −3 mV, respectively.

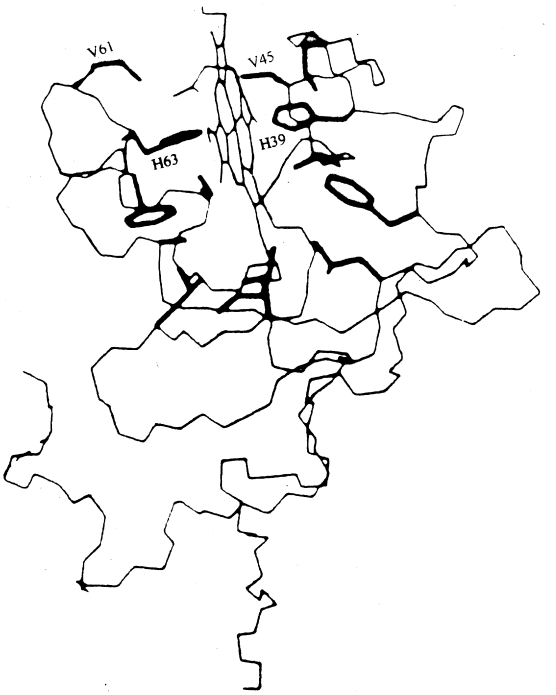


Fig. 1. Stereo view of the heme group, the peptide backbone and the 14 side chains (heavy lines) which are in contact with the heme in the molecule of bovine cytochrome b_5.

2. Materials and methods

2.1. Materials

T4 DNA polymerase, ligase, kinase and DNA restriction endonucleases were purchased from Biolabs. IPTG and X-gal were bought from Sigma.

[γ-^{32}P]dATP were obtained from Amersham. All chemicals were of reagent grade. The pUC19 plasmid containing the synthesized gene encoding the tryptic bovine hepatic cytochrome b_5 (82 residues in length) was a kind gift from Professor Mauk [13].

2.2. Mutagenesis, expression and purification of cytochrome b_5

Site-directed mutagenesis of the gene coding for trypsin-solubilized cytochrome b_5 was accomplished as described by Zoller and Smith [14]. Three 21-base oligonucleotides were synthesized and purified. The codon of Val45 (GTC) was changed into GAC (Glu), TAT (Tyr) and CAT (His). The mutated DNA sequences were confirmed using dideoxy chain termination method [15]. The mutated genes from M13mp19RF DNA were ligated into pUC19 vector and transformed into *E. coli* JM83. The expression and purification of mutant cytochrome b_5 were performed according to the literature [13].

2.3. Electrospray mass spectrometry

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

2.4. Redox potential

The redox potentials were measured by differential pulse voltammetry (DPV). The working electrode was a 2-mm-diameter gold disc electrode, with a platinum wire-coil as counter electrode and Ag | AgCl | KCl (3 M) as reference electrode. The polishing and modification of the working electrode were accomplished according to the published method [16]. Protein solutions were prepared in Hepes buffer (1 mM), and were degassed by bubbling gently with nitrogen for 15 min. After degassing, the protein was kept under a constant stream of nitrogen. Experiments were performed at 25 ± 1°C using a PAR M273 potentiostat galvanostate (PAR Princeton, NJ, USA) controlled by PAR M270 software. The cell consists of a conventional three-electrode system with a small volume sample ($V \sim 0.5$ ml). The pulse height and width are 10 mV and 50×10^{-3} s, respectively. The scan rate is 1 $mV \cdot s^{-1}$. The $E_{1/2}$ value, which approximately equals the formal reduction potential, was obtained by applying the Parry–Osteryoung relationship: $E_p = E_{1/2} - \Delta E/2$, that relates the peak potential E_p to the half-wave potential $E_{1/2}$ for a given pulse amplitude ΔE [17]. All the experiments were repeated several times and the values of the reduction potentials were found to be reproducible within ± 3 mV. The potentials reported in this paper are all referenced to NHE.

2.5. UV-Visible spectrometry

All denaturations were monitored spectrophotometrically using a Hewlett-Packard 8452A diode array spectrometer controlled by commercial software unless otherwise stated. The protein concentration was calculated assuming an extinction coefficient of 117 mM^{-1} cm^{-1} at 412.5 nm.

2.6. Thermal denaturation

Proteins were dissolved in 100 mM sodium phosphate buffer (pH 7.0) with a concentration of 5 μM. The ferrocytochrome b_5 was prepared by addition of sodium dithionite to the protein solution [18]. The spectra were recorded over a temperature range of 25–90°C and for each experiment the solution was incubated for a period of 15 min to ensure that equilibrium had been reached. The temperature was determined directly at the cuvette holder and was maintained within ± 0.1°C.

2.7. Urea and ethanol denaturation

All experiments were carried out at a protein concentration of 5 μM and 100 mM sodium phosphate buffer (pH 7.0). Each sample contained

different concentrations of urea (ranging from 0 to 9 M) or ethanol [ranging from 0 to 63% (v/v)]. Protein solutions were allowed to equilibrate with denaturants for 20 h at room temperature until the absorbance at 412 nm had stabilized and spectra were recorded from 200 to 800 nm. A UV240 spectrophotometer was used to monitor ethanol denaturation.

2.8. Acid denaturation

Mutants and wild type Cyt b_5 were dissolved in 100 mM KAc–HCl buffer (pH value from 1.2 to 5.3) or sodium phosphate buffer (pH value from 5.5 to 7.0). Protein concentration is 5 μM. The protein solution was incubated for 3 h at room temperature before recording the spectrum.

2.9. Analysis of heat and urea denaturation curves

The denaturation of cytochrome b_5 by heat or urea can be described by a two-state mechanism [19,20]

$$N \Leftrightarrow D \tag{1}$$

in which only the native (N) and the denatured (D) state of cytochrome b_5 exist in the solution at certain conditions. The equilibrium constant K_D and the free energy of the denaturation ΔG_D can be calculated from

$$K_D = f_D/f_N = (A_N - A)/(A - A_D), \tag{2}$$

$$\Delta G_D = -RT \ln K_D \tag{3}$$

where f_D and f_N are the fractions of cytochrome b_5 in the denatured and native state, respectively. The value A is the measured absorbance at 412 nm, A_N is the absorbance of the native protein (protein at room temperature or no urea) and A_D is the absorbance of the denatured protein (protein at 85°C or 8 M urea).

For the denaturation caused by urea, ΔG_D can be calculated from

$$\Delta G_D = \Delta G_D^0 - m_D[\text{urea}] \tag{4}$$

where [urea] is the concentration of urea and m_D is the slope of the plot of ΔG_D against urea concentration over the linear region around the transition concentration of urea and ΔG_D^0 is the free energy of heme dissociation at room temperature and zero concentration of denaturant. The difference in the free energy of heme dissociation between the wild type and the mutant proteins can be calculated from the extrapolated free energy of heme dissociation according to the following equation:

$$\Delta(\Delta G_D^0) = \Delta G_{D(WT)}^0 - \Delta G_{D(\text{mutant})}^0 \tag{5}$$

However, since such extrapolation is subjected to 30% errors [20], the more accurate way is to calculate the difference in free energy at the midpoint of heme dissociation [$\Delta(\Delta G_D^{50\%})$] by the following equation [20]:

$$\Delta(\Delta G_D^{50\%}) = \langle m \rangle [C_m(\text{mutant}) - C_m(\text{WT})] \tag{6}$$

in which $\langle m \rangle$ is the average value of slope, m_D for the wild type and mutant proteins, and C_m(mutant) and C_m(WT) are the values for the midpoints of heme dissociation for the mutant and wild type proteins.

For heat denaturation, Eq. (3) can also be written as:

$$\Delta G_D = -RT \ln K_D = \Delta H_m + T\Delta S_m \tag{7}$$

A plot of $\ln K_D$ against $1/T$ (or of ΔG_D against T) allows estimates of the enthalpy ΔH_m and entropy ΔS_m from the linear region around the transition temperature T_m (under this temperature, $f_N = f_D$). Also, the $\Delta(\Delta G_D^{50\%})$ can be estimated from [18,21]:

$$\Delta(\Delta G_D^{50\%}) = \langle \Delta S_m \rangle [T_m(\text{mutant}) - T_m(\text{WT})] \tag{8}$$

where $\langle \Delta S_m \rangle$ is the average of the ΔS_m values for the wild type and mutant proteins.

3. Results

3.1. Mutagenesis, expression and purification of cytochrome b_5

Site-directed mutagenesis were performed using three synthesized oligonucleotides. The positive clones were selected by hybridization with the oligonucleotides labeled with γ-^{32}P and confirmed by DNA sequencing. The mutated Cyt b_5 genes were cloned into pUC19 vector and transformed into JM83 successfully. The yields of purified protein of wild type, V45Y, V45H and V45E of cytochrome b_5 were 15, 10, 9 and 5 mg/l of culture solution. The purified proteins showed a single band on SDS-PAGE and were further confirmed by ES-MS. The measured molecular weights are: 9461.5 ± 1.3 (wild type); 9527.9 ± 2.69 (V45Y); 9499.83 ± 2.68 (V45H); and 9490.41 ± 2.79 (V45E) which correspond to the molecular weights calculated from amino acid compositions of apo-cytochrome b_5 (9461.3, 9525.3, 9499.3, and 9491.3 Da, respectively). These results confirm the successful mutagenesis.

3.2. Redox potential

The redox potential of an electron-transfer protein provides the driving force for the biochemical reaction. In an attempt to evaluate the effect of mutation on their functions, it is often desirable to measure the redox potential of the protein and its site-directed mutants. In this work, redox potentials of cytochrome b_5 and its mutants were measured using differential pulse voltammetry. Since these experiments were carried out in the absence of promoters such as polylysine, Ca^{2+} and Mg^{2+} ions etc., these results should directly reflect the redox property of cytochrome b_5. The redox potentials of mutant V45Y, V45H, V45E and wild type of cytochrome b_5 are -35 mV, 8 mV, -26 mV and -3 mV, respectively.

3.3. Thermal stability studies

Absorption spectra of the thermal denaturation of oxidized and reduced state of cytochrome b_5 V45H are shown in Fig. 2a,b. The maximum absorbance of Soret band for the oxidized and reduced state are at 412 and 424 nm, respectively. According to the two-state mechanism, it is reasonable to evaluate the thermal stability by changes in Soret peaks at 412 and 424 nm [18,20–22]. The spectra show a progressive decline in the magnitude of the absorbance between 300 and 600 nm with increasing temperature. Most noticeably, as the temperature reaches a certain value, the absorbance of Soret band decreases dramatically and a shift from 412 nm to 394 nm for oxidized state and from 424 nm to 407 nm for reduced state are observed. An isobestic point is observed which corroborates the two-state mechanism. The normalized figures as a function

Table 1
Thermodynamic data for thermal denaturation of cytochrome b_5[a]

Cytochrome b_5	T_m (°C)	ΔT_m (°C)	ΔH_m (kJ/mol)	ΔS_m (J/mol K)	$\Delta(\Delta G_D^{50\%})$ (kJ/mol)
Oxidized WT	66.7	–	377	1110	–
Oxidized V45Y	60.5	−6.2	351	1051	−6.4
Oxidized V45H	61.6	−5.1	367	1092	−5.6
Oxidized V45E	56.8	−9.9	380	1151	−11.2
Reduced WT	77.6	–	358	1022	–
Reduced V45Y	72.1	−5.5	469	1353	−6.5
Reduced V45H	72.1	−5.4	394	1142	−5.8
Reduced V45E	69.0	−8.6	332	971	−8.6

[a] 100 mM sodium phosphate buffer, pH 7.0.

of temperature are shown in Fig. 2c,d for both the wild type and mutant proteins. Transition temperature (T_m), enthalpy (ΔH_m), entropy (ΔS_m) and the free energy difference ($\Delta\Delta G_D^{50\%}$) are summarized in Table 1. These thermodynamic parameters correspond to the overall heme release and protein unfolding process. Based on these values, it is obvious that the wild type and mutant forms of ferrocytochrome b_5 are significantly more heat stable than the ferricytochrome b_5. The relative heat stability has the order: WT > V45Y ~ V45H > V45E, being both in the oxidized and reduced state.

3.4. Urea denaturation

The denaturation absorption spectra of mutants and wild type cytochrome b_5 induced by

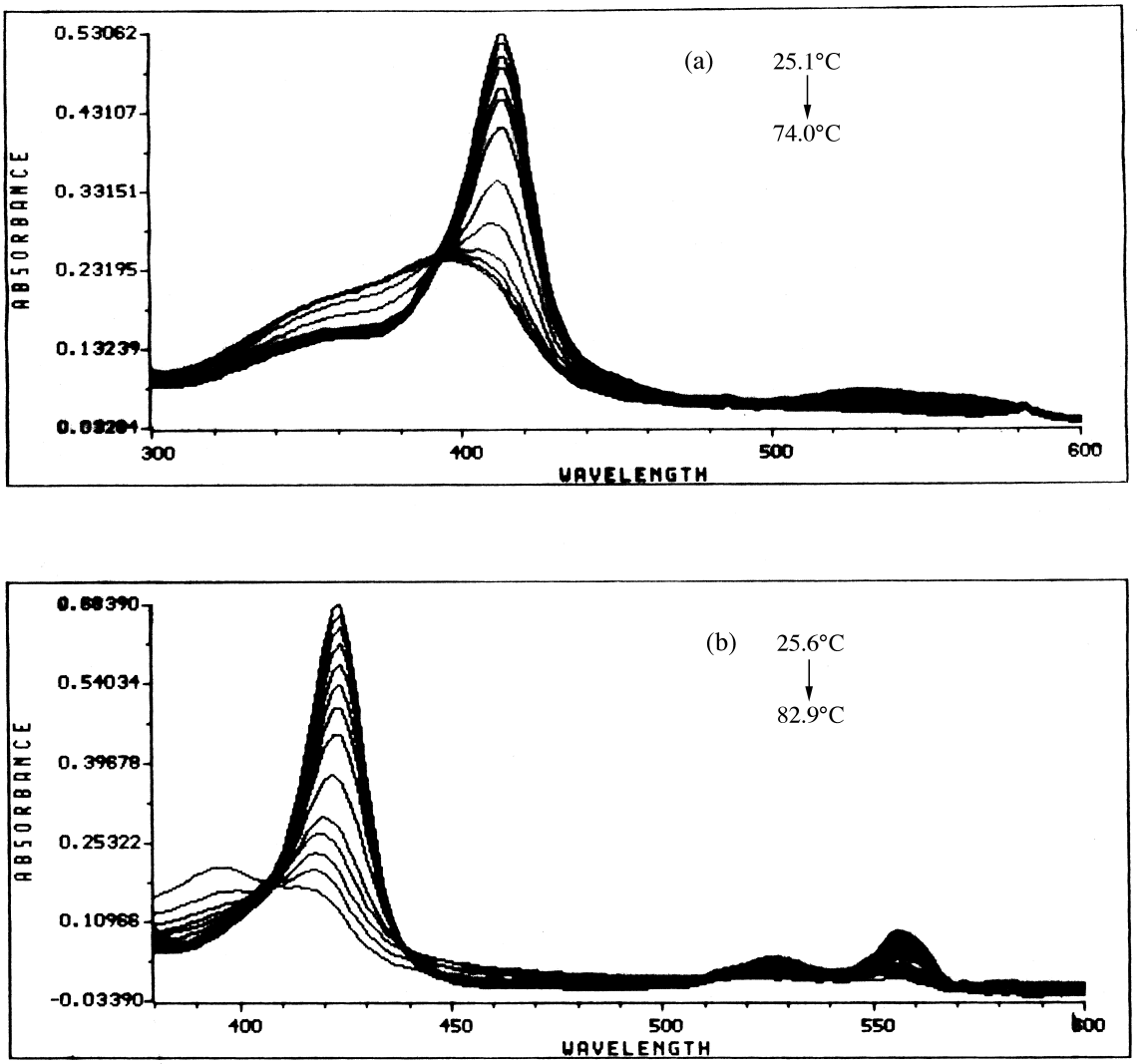
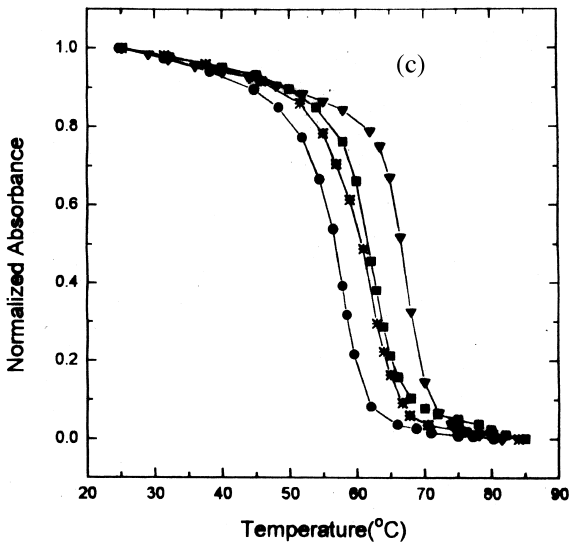


Fig. 2. Equilibrium studies of thermal denaturation of the wild type and Val45 mutants of bovine cytochrome b_5. (a) Absorbance spectra of the ferricytochrome b_5 V45H at temperature of 25.1, 36.0, 47.2, 52.0, 56.5, 58.0, 60.0, 62.2, 64.0, 66.1, 68.0, 71.0 and 74.0°C. (b) Absorbance spectra of the ferrocytochrome b_5 V45H at temperature of 25.6, 37.0, 49.0, 56.0, 63.0, 67.1, 69.0, 72.0, 74.1, 76.0, 78.2, 81.1, 82.0, 82.9°C. (c) Normalized changes in absorbance at 412 nm with increasing temperature for oxidized proteins. (d) Normalized changes in absorbance at 424 nm with increasing temperature for reduced proteins. Symbols correspond to wild type (▼), Val45Tyr (*), Val45His (■), Val45Glu (●).



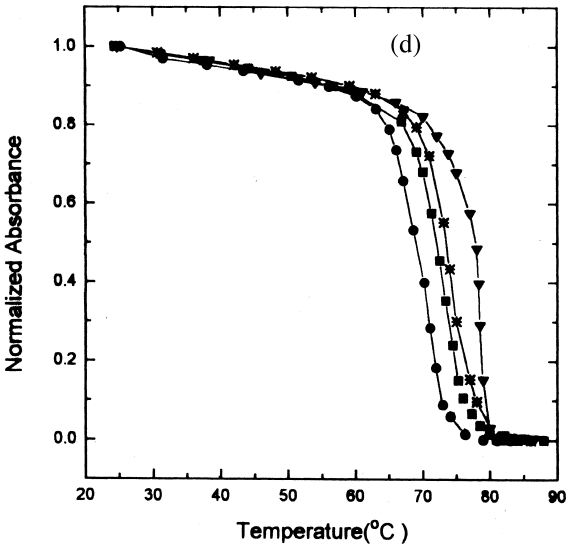


Fig. 2. (*Continued*)

urea change in the same way as that of heat denaturation. Fig. 3a shows the changes in the intensity of Soret band of cytochrome b_5 V45Y upon exposure to increasing concentrations of urea. An isobestic point at 398 nm in the spectra confirms that it is a two-state module which can be analyzed as described in Section 2. The wild type protein and other mutants follow similar pattern with increasing concentrations of urea (figure not shown). From Eq. (2) and Eq. (4), denaturation constants can be calculated. Table 2 shows the transition concentration of urea (C_m) and the difference in free energy between the wild type and mutant proteins ($\Delta\Delta G_D^{50\%}$). According to these values, it can be concluded that all three mutants are less stable towards urea than the wild type. V45E is the most unstable mutant, V45Y is the most stable among three mutants and the stability of V45H lies in the middle.

3.5. Ethanol and acid denaturation

Changes in the absorption spectrum of the cytochrome b_5 V45E mutant upon exposure to increasing concentration of ethanol and pH values are shown in Fig. 5a and Fig. 4a. It is conspicuous that the profiles of these spectra are very different from those of spectra using urea or heat as denaturants. No isobestic points are observed. As concentrations of ethanol increase, the maximum absorption peak shifts from 412 nm to 404 nm and finally to 400 nm. When these mutants are exposed to acid, absorption of 412 nm decreases drastically with pH decreasing. In the end, a broad peak around 372 nm is observed in all mutants and wild type of Cyt b_5. In order to compare the results of different mutants, normalized absorbance are calculated from $(A - A_{1.2})/(A_{7.0} - A_{1.2})$ for acid denaturation and $(A - A_{63\%})/(A_0 - A_{63\%})$ for ethanol denaturation, where A is the measured absorbance of Soret band, $A_{7.0}$ and $A_{1.2}$ refer to the absorbance of proteins at pH value of 7.0 and 1.2, and A_0 and $A_{63\%}$ refer to the absorbance of proteins at 0 and 63% concentrations of ethanol, respectively. Fig. 4b and Fig. 5b show the normalized absorbance in the intensity of 412 nm of cytochrome b_5 V45E with increase of the pH or ethanol concentrations. The pH midpoint of mutants (normalized absorbance equal to 0.5) towards acid are: 4.7 (V45Y); 5.0 (V45H); 5.1 (V45E); and 4.0 (WT). The concentration midpoint of mutants V45Y, V45H, V45E and wild type of cytochrome b_5 towards ethanol correspond to 29%, 30%, 29% and 35%, respectively. It can be seen that all

Table 2
Urea denaturation of bovine cytochrome b_5[a]

Cytochrome b_5	C_m (mol/l)	m_D (kJ l/mol^2)	$\Delta(\Delta G_D^{50\%})$ (kJ/mol)
Wild type	6.8	−3.7	–
Val45Tyr	5.6	−3.1	−4.0
Val45His	5.1	−4.4	−6.9
Val45Glu	4.8	−4.3	−8.0

[a]100 mM sodium phosphate buffer, pH 7.0.

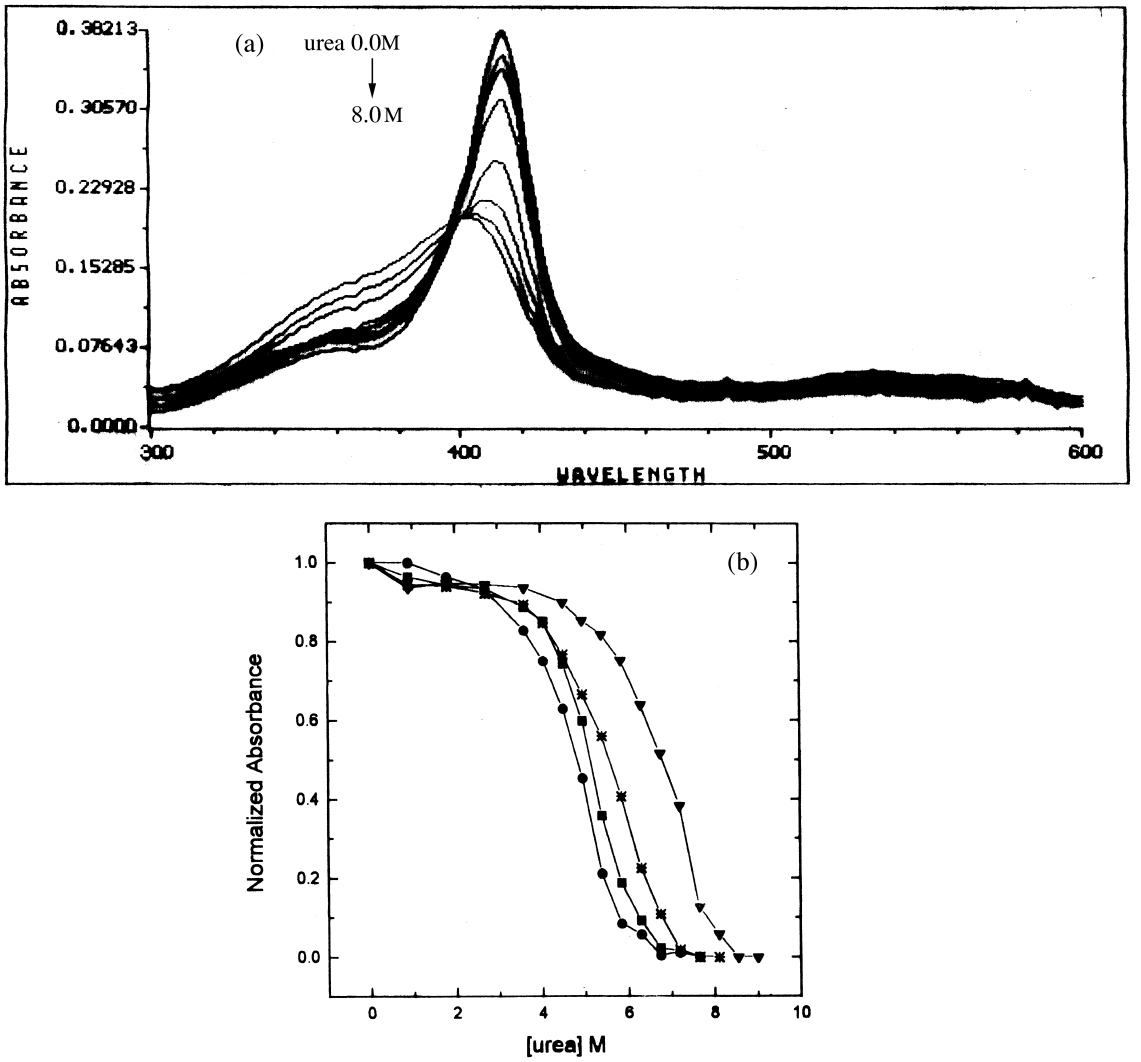


Fig. 3. Equilibrium studies of urea denaturation of the wild type and Val45 mutants of bovine cytochrome b_5. (a) Absorbance spectra of the V45Y mutant at different urea concentrations (0–8 M); (1 M increment). (b) Normalized changes in absorbance of the V45Y mutant measured at 412 nm as a function of the urea concentration. Symbols correspond to wild type (▼), Val45Tyr (*), Val45His (■), Val45Glu (●).

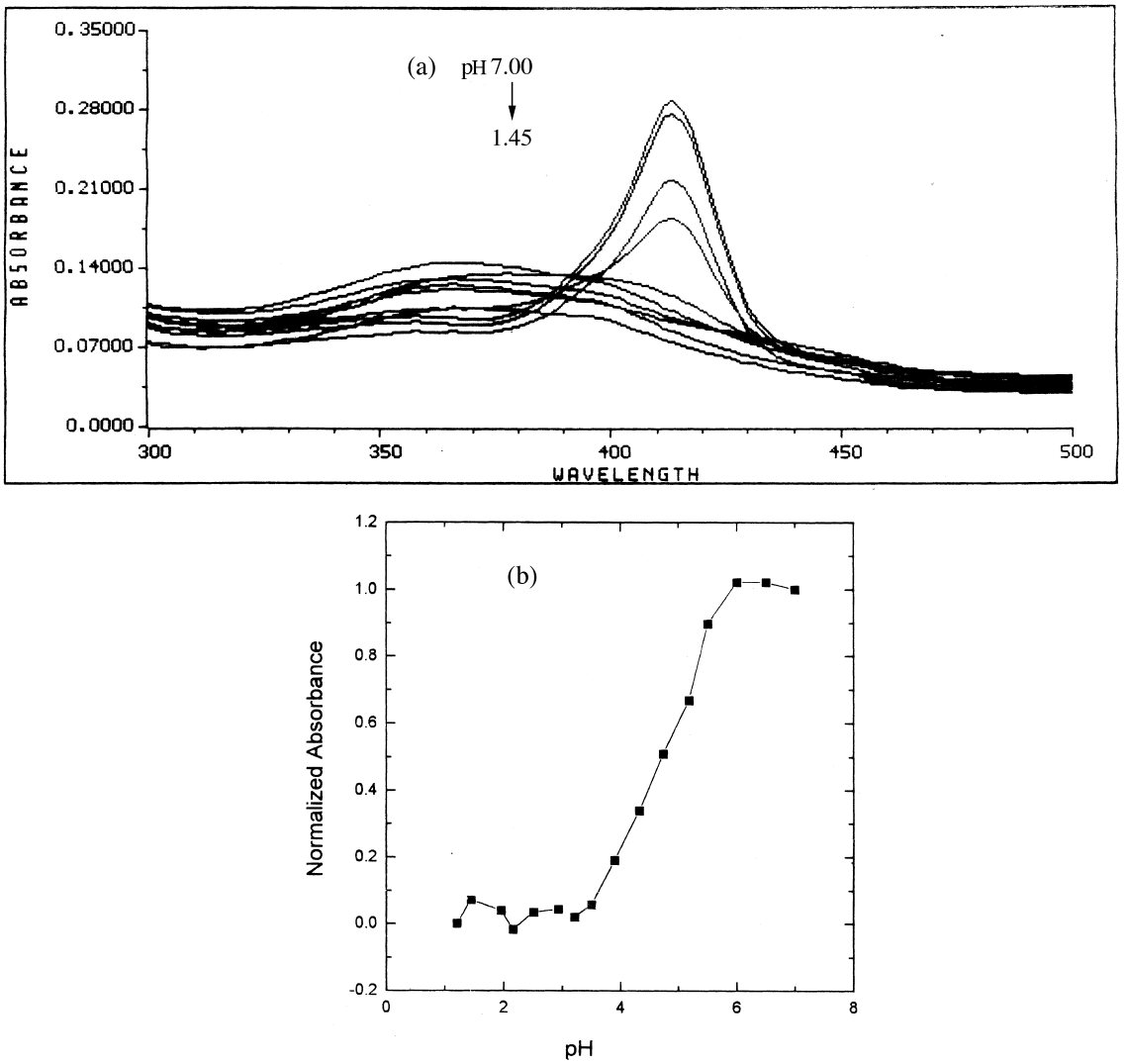


Fig. 4. Acid denaturation of cytochrome b_5. (a) Absorbance spectra of the V45Y mutant at different pH (1.45, 1.96, 2.16, 2.51, 3.21, 3.90, 4.74, 5.18, 6.50, 7.00). (b) Normalized changes in absorbance at 412 nm of the V45Y mutant as a function of pH.

three mutants show similar stability and a slight less stable than the wild type protein.

4. Discussion

4.1. Protein stability

The method of site-directed mutagenesis is ideal for elucidating the roles of individual amino acid residues play in modulating protein stability. It is well known that the effect of a substitution depends on the nature of the substitute and its structure contexts. Substitution on a mobile exposed site usually has little effect on stability of the protein [2,23], since the conformational flexibility often allows the protein to minimize the effects of a potentially deleterious substitution by locally readjusting to give an alternative structure

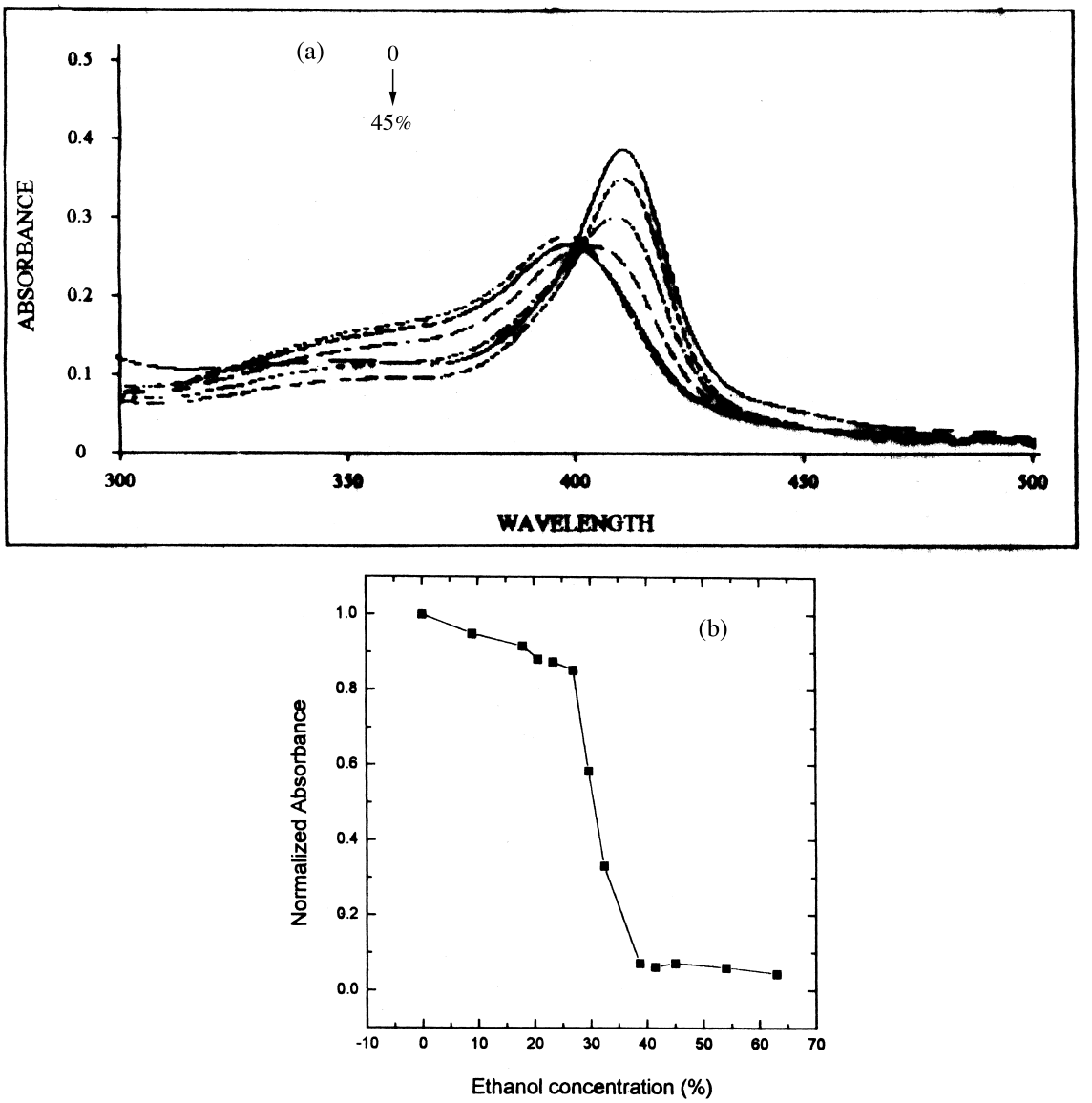


Fig. 5. Ethanol denaturation of cytochrome b_5. (a) Absorbance spectra of the V45E mutant at ethanol concentration of 0, 23, 30, 32, 36, 45%. (b) Normalized changes in absorbance at 412 nm of the V45E mutant as a function of ethanol concentration.

that is energetically comparable to the wild type [24,25]. But generally speaking, the relatively rigid amino acids [24] and the residue inaccessible to solvent [22,26,27] make significant contributions to stability. The difference in stability can be due to changes in charge, size, polarity, hydrophobicity, van der Waals contacts or hydrogen bonding capacity, etc.

We have examined the effect of three Val45 mutants on denaturation induced by thermal, urea, acid and ethanol. It is noticeable that although all three mutants show lower stability with respect to the wild type protein towards different denaturants, the stability order changes in different manner. It appears that different behaviors resulted from different denaturing mechanism.

Generally speaking, there are two factors contributing to the stability of the heme protein: one is the secondary and tertiary structure stability of the polypeptide chain, another one is the binding stability of the heme group to the polypeptide. The thermal denaturation of cytochrome b_5 may involve the loss or partial dissociation of the heme from the polypeptide and the iron ion changes from low spin state to high spin state [18]. Xue et al. [28] have further confirmed that with increasing temperature the heme group dissociated from the protein with the concordant polypeptide unfolding. Also, when Cyt b_5 is exposed to the increasing concentration of urea, the heme will leave the accompanying heme pocket by destroying the globular structure of the molecule.

A series of studies show that the hydrophobic effect is an important stabilizing force in protein folding [29,30] and an important driving force [31]. Since residue Val45 contacts the heme group and is a part of the heme hydrophobic pocket, the relative hydrophobicity of the substituting residue must be considered. Based on the criteria proposed by Caffrey [32] replacement of tyrosine, histidine and glutamic acid for valine would generate $+0.4$, -1.6, -2.7 kJ/mol of hydrophobic free energy changes [$\Delta(\Delta G)$] which are consistent with the observed stability of the proteins. Obviously, the changes of hydrophobic free energy is a dominant factor contributing to stability of the Val45 mutants upon heat and urea denaturation. As we know, replacement of a hydrophobic residue always leads to altered protein stability. For example, the substitution of the side chains of *Rb. capsulatus* Pro30 and Trp59 by alanine and tyrosine (i.e. P30A and W59Y) resulted in lower stability [33,34]. Our results corroborated the view that for globular hemoproteins the hydrophobic effect is the major factor in stabilizing the folded structure.

By comparing the relative stability of the three Val45 mutants, it is found that the V45E mutant is most unstable. This can be related to its conformational changes. According to the X-ray structure analysis of cytochrome b_5, the stabilization of helix III around the heme is balanced by its two faces: the hydrophilic one which is faced to the surface of the protein and the hydrophobic one which makes up the inner wall of the heme hydrophobic pocket. Polar residues such as Glu43, Glu44, Arg47 and Glu48 are located on the surface of the molecule, while the non-polar residues such as Val45 and Leu46 of this helix are on the hydrophobic pocket contacting with the heme. When Val45 was substituted by Glu, the carboxyl group of glutamic acid was forced away from the pocket due to its bigger $\Delta(\Delta G)$ and flexibility. This situation is also observed in the V45L mutant of outer mitochondria membrane cytochrome b_5 [35] in which the longer leucine side chain points away from the heme. After replacement of Val by Glu, the sequence of residues on the helix III was changed to Gly41–Gly42–Glu43–Glu44–Glu45. These arrangements surely change the balance of the helix III, leading to loosing of the helix structure. Thus, the protein stability of the V45E mutant changes much more than the other two mutants. Since the lower difference of hydrophobic free energy, $\Delta(\Delta G)$, and the larger volume of the tyrosine make the phenylhydroxyl group of the tyrosine still sitting inside the hydrophobic pocket, the minor perturbation on the local conformation of the heme pocket results in V45Y mutant being less stable than the wild type and more stable than the other two mutants.

The mechanisms of denaturation towards acid and organic solvent are much more complicated. Classical theory [36] predicts that with a decrease of the pH from neutral region to approximately pH 2, the protein becomes maximally positively charged. The resulting intermolecular repulsion between the positively charged groups leads to the unfolding of the protein. Ikeda et al. [37] had measured the optical and EPR spectra of cytochrome b_5 from pig liver in the pH range of 3.5–12.0 and found that the spin state of iron ion changed from low spin to high spin below pH 4.0. Such changes can be attributed to the heme release from the protein. So, it can be assumed that with increase of pH, intramolecular repulsion of cytochrome b_5 results in loosing of the structure of the heme crevice. Consequently, partial disrup-

tion of the hydrophobic environment and exposing of some peptides to the solvent lead to the heme leaving. Thus, the absorbance of Soret band decreased dramatically. No isobestic point was observed indicating the denaturing process undergoes a non-two-state mechanism. It seems reasonable to understand that the Val45 mutants show similar stability towards acid denaturation. Their lower stability with respect to the wild type may be attributed to the local conformational change of mutants.

Ethanol-induced denaturation has a more complex mechanism rather than a simple two-state module. Non-covalent interactions such as hydrogen bonds and hydrophobic contacts are considered to be responsible for the stability of the protein induced by alcohol. As we know, in order to construct a stable structure in water, the nonpolar residues are often buried inside the protein and prone to form a hydrophobic core [38]. On the contrary, the polar residues stay on the surface of the protein. So a stable protein is always represented by a low dielectric core (usually, the dielectric constant inside the protein is estimated to be approximately 2–4 [39]) embedded in the high dielectric solvent (for example, the dielectric constant of water is 78.54). In the case of cytochrome b_5, 14 hydrophobic residues make up the heme hydrophobic pocket and 13 charged residues lay on the surface. When it is exposed to the ethanol–water solvent, the lower polarity and dielectric constant of the solvent decrease the ability of forming the hydrophobic heme pocket, making the hydrophobic core more loose. Thus, the binding of the heme to the heme hydrophobic pocket will be decreased, even destroyed. On the other hand, high concentration of ethanol (dielectric constant, $\varepsilon_{\text{ethanol}} = 24.55$ [40]) often weaken hydrogen bonds between the solvent and protein, causing the hydrogen bonds within the polypeptide chain to be comparatively stronger [41,42]. This effect can be considered as a stabilizing factor of the helix structure of the protein. However, on the whole, it can not compensate the influence of the hydrophobic interaction. Therefore, the ultimate result is the destabilization of the folded state, making the heme leave the pocket more easily. The similar stability of all three mutants towards ethanol is likely due to the fact that compared to the changes of dielectric constant of the solvents, the natural alteration of the replaced residues is almost meaningless. In comparison with the wild type, the lower protein stability observed in the mutant proteins probably results from the local conformational changes. The detailed mechanism of alcohol-induced denaturation of Cyt b_5 is currently being investigated using fluorescence spectroscopy and circular dichroism. The results indicate that with increase of alcohol concentration, the heme group dissociated from the protein accompanied with the concordant polypeptide unfolding.

4.2. Redox potential

The cytochromes are a group of ubiquitous heme proteins that are involved in a series of important electron transfer chains in biological systems. Their redox potential span an enormous range from -150 mV to $+300$ mV [43]. It is the difference in the redox potentials that makes the electron transfer along the correct direction and can be regarded as the driving force for the biochemical reactions and the indicator of biological functions. Thus, it is very attempting to evaluate the factors responsible for the diversity of redox potentials in heme proteins. A large number of theoretical and experimental work have been focused on the role of charges: at the heme exposed surface [2,16,39,44,45]; variable dielectric matrix [46,47]; axial imidazole ligand plane orientation [48–51]; hydrophobicity of the heme pocket [52]; and hydrogen bonding to the axial imidazole ligands [53], etc. All these results demonstrate that factors contributing to the reduction potential of cytochrome b_5 are comprehensive.

In order to provide more information, three Val45 mutants were designed and obtained by site-directed mutagenesis. As we mentioned previously, since the crucial location of residue Val45, it is reasonable to find changes of the redox potential of the proteins. The redox potentials of mutants V45Y, V45H, V45E and the wild type are -35 mV, 8 mV, -26 mV and -3 mV,

Table 3
Reduction potentials of Val45, Val61 and Phe35 mutants of cytochrome b_5

Protein	$E_{1/2}$ (mV)	Protein	$E_{1/2}$ (mV)	Protein	$E_{1/2}$ (mV)
WT	−3	WT[a]	−10	WT[b]	−1
V45Y	−35	V61Y[a]	−33	F35Y[b]	−66
V45H	8	V61H[a]	12	F35L[b]	−28
V45E	−26	V61E[a]	−25	F35L[b]	−51

[a]See Xue et al. [45].
[b]See Yao et al. [52].

respectively. A very suspicious thing that puzzles us is why the V45Y mutant presents the most negative shift of redox potential; on the contrary, the V45E mutant shows the most destabilization effect on the protein? As we concluded before [22,44,54] the redox potential of cytochrome b_5 is mainly dominated by three factors: the direct perturbation (binding) on the heme (including the axial ligands, the interaction between propionate groups and external ions or groups); the directed contact between the heme and nearby groups; and the charge density and distribution around the heme [45]. As described above, the lower difference in hydrophobic free energy $\Delta(\Delta G)$ and the larger volume of the tyrosine compared with glutamic acid make the phenylhydroxyl group of V45Y still sitting inside the hydrophobic pocket. This situation is similar to the F35Y mutant of cytochrome b_5 ($E_{1/2} = -64$ mV, shown in Table 3), in which this conformation was proved by X-ray structure analysis and NMR solution structure study (unpublished data). The close contact between the heme and the hydroxyl group of tyrosine affects the reduction potential seriously. An additional evidence is that since the long carbon stem of the lysine residue is swinging out of the hydrophobic pocket in cytochrome b_5 Y74K mutant, there is no change at all on redox potential and stability of the protein by the mutation [20]. On the contrary, the carboxyl group of glutamic acid in the mutant V45E was forced to move away from the pocket due to its bigger $\Delta(\Delta G)$ and flexibility, accounting for its less influence on the redox potential.

As discussed previously, the charge density and distribution around the heme is also a main factor to modulate the reduction potential of the heme protein. In the case of a negatively charged protein such as cytochrome b_5, an increase of positive charge at the heme exposed surface always stabilized reduced state of the heme iron, making the redox potential shift positively. On the contrary, a decrease of positive charge destabilized the reduced state of the heme iron ion, making the reduction potential negatively shift. This was discussed in the cytochrome b_5 V61 mutants in details [45]. When Val45 is substituted by the negatively charged residue glutamic acid, it consists of other negative charge areas with Glu43, Glu44 and Glu48 on the left of the heme, causing its redox potential to shift negatively approximately 23 mV. Just like the V45E mutant, the side chain of the V45H mutant also shifts away a little bit from the heme pocket, the introduction of a positively charged group resulted in a positive shift of 11 mV with respect to wild type. This is exactly the same situation as in the case of mutation at Val61 of cytochrome b_5, where the redox potentials of the V61E mutant shows a shift to a more negative value and the mutants V61H and V61K present a positive shift (shown in Table 3).

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