Effects of Some Charged Amino Acid Mutations on the Electron Self-Exchange Kinetics of Cytochrome b_5

Dejian Ma, Yibing Wu, Chengmin Qian, and Wenxia Tang*

State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, P.R. China

Yun-Hua Wang, Wen-Hu Wang, Jun-Xia Lu, Yi Xie, and Zhong-Xian Huang*

Chemistry Department, Fudan University, Shanghai 200433, P.R. China

Received May 25, 1999

Two mutants of trypsin-solubilized bovine liver cytochrome b_5 (cyt b_5) have been prepared to elucidate the function of charged residues near the heme exposed edge in the electron self-exchange kinetics of cyt b_5. The Glu44, Glu56, and Asp60 were mutated into alanines in mutant I (E44A/E56A/D60A) while Glu44, Glu48, Glu56, and Asp60 were mutated into alanines in mutant II (E44A/E48A/E56A/D60A). The electron self-exchange rates of cyt b_5 mutants I and II have been measured as a function of temperature and ionic strength using the one-dimensional ^{1}H NMR saturation transfer method. Under the condition of $\mu = 0.10$ M and [cyt b_5] = 0.50 mM, the rate constant of the cyt b_5 mutant I is $(4.0 \pm 0.4) \times 10^3$ M^{-1} s^{-1} at 20 °C. The value rises to $(7.2 \pm 0.7) \times 10^3$ M^{-1} s^{-1} at 35 °C; data from 20 to 35 °C gave $\Delta H^{\ddagger} = 6.8 \pm 0.8$ kcal mol^{-1} and $\Delta S^{\ddagger} = -19 \pm 3$ eu. The rate constant for cyt b_5 mutant II is $(8.4 \pm 0.7) \times 10^3$ M^{-1} s^{-1} at 20 °C with $\mu = 0.10$ M and [cyt b_5] = 0.60 mM. The value rises to $(17 \pm 1) \times 10^3$ M^{-1} s^{-1} at 35 °C, $\Delta H^{\ddagger} = 7.9 \pm 0.9$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -14 \pm 4$ eu. The rate constant for cyt b_5 mutants increases with increasing of ionic strength. The values of the rate constants of cyt b_5 mutants extrapolated to infinite ionic strength, k_{inf}, are 2.8×10^5 and 1.1×10^5 M^{-1} s^{-1} for mutant I and mutant II, respectively. The protein dipole moment projections through the heme exposed edge for two mutants were obtained from a fit of ionic strength dependence of self-exchange rate constants using van Leeuwen's approach. The dipole moment projections were −220 D and −254 D for oxidized and reduced forms of cyt b_5 mutant I, and −134 D (oxidized) and −159 D (reduced) for cyt b_5 mutant II. In terms of Marcus theory, the reorganization energies of cyt b_5 mutant I and mutant II have been estimated to be 1.2 and 1.3 eV, respectively. The association constants for the electron self-exchange reactions of mutant I and mutant II are 0.33 and 1.5 M^{-1}, respectively. Based on above results, the recognition and electron self-exchange mechanism between oxidized and reduced states of cytochrome b_5 was discussed.

Introduction

Metalloproteins undergo electron-transfer reactions, which are fundamental phenomena in the mitochondrial respiratory chain, photosynthesis, oxidative phosphorylation, and many other processes. The electron-transfer rate constants depend on many factors, including the distance between electron donor and acceptor centers, thermodynamic driving force, reorganization energy, nature of the intervening medium, etc.[1−6] Site-directed mutagenesis method in conjunction with NMR techniques has proved to be a useful approach to study electron-transfer reactions between metalloproteins.[7]

The study of the electron self-exchange reaction is one effective way to demonstrate the electron-transfer mechanism because the thermodynamic driving force is zero in self-exchange kinetics, this simplifies the interpretation of the intrinsic electron-transfer process.[8−13] Cytochrome b_5 is a small, structurally well-characterized protein.[14−17] In addition, single and multisite protein variants can be prepared in order to interpret those structural elements that affect the rate of electron

* Corresponding authors, W. Tang: Nanjing University, fax, +86 25 3314502, e-mail, wxtang@netra.nju.edu.cn. Z.-X. Huang: Fudan University, fax, +86 21 65641740, e-mail, zxhuang@fudan.edu.cn.

(1) Scott, R. A.; Mauk, A. G.; Gray, H. B. *J. Chem. Ed.* **1985**, *62*, 932−938.
(2) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160−167.
(3) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(4) McLendon, G.; Hake, R. *Chem. Rev.* **1992**, *92*, 481−490.
(5) Twitchett, M. B.; Ferrer, J. C.; Siddarth, P.; Mauk, A. G. *J. Am. Chem. Soc.* **1997**, *19*, 435−436.
(6) Dixon, D, W.; Hong, X.; Woehler, S. E.; Mauk, A. G.; Sishta, B. P. *J. Am. Chem. Soc.* **1990**, *112*, 1082−1088.
(7) Mauk, A. G.; Mauk, M. R.; Moore, G. R.; Northrup, S. H. *J. Bioenerg. Biomembr.* **1995**, *27*, 311−330.
(8) Dixon, D. W.; Hong, X.; Woehler, S. E. *Biophys. J.* **1989**, *56*, 339−351.
(9) Kyritsis, P.; Dennison, C.; Ingledew, W. J.; McFarlane, W.; Sykes, A. G. *Inorg. Chem.* **1995**, *34*, 5370−5374.
(10) Im, S. C.; Zhuang-Jackson, H. Y.; Kohzuma, T.; Kyritsis, P.; McFarlane, W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1996**, 4287−4294.
(11) Gaillard, J.; Zhuang-Jackson, H. Y.; Moulis, J. M. *Eur. J. Biochem.* **1996**, *238*, 346−349.
(12) Legrand, N.; Bondon, A.; Simonneaux, G. *Inorg. Chem.* **1996**, *35*, 1627−1631.
(13) Andrew, S. M.; Thomasson, K. A.; Northrup, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 5516−5521.
(14) Argos, P.; Mathews, F. S. *J. Biol. Chem.* **1975**, *250*, 747−751.
(15) Mathews, F. S.; Levine, M.; Argos, P. *J. Mol. Biol.* **1972**, *64*, 449−464.
(16) Mathews, F. S.; Czerwinski, E. W. *The Enzymes of Biological Membranes*; Martonosi, A. E., Ed.; Plenum Press: New York, 1976; Vol. 4, pp 143−197.
(17) Mathews, F. S. *Prog. Biophys. Mol. Biol.* **1985**, *45*, 1−56.

10.1021/ic990607k CCC: $18.00
Published on Web 11/18/1999

self-exchange reaction of cytochrome b_5.[18,19] To elucidate the function of charged residues near the exposed heme edge in the electron self-exchange kinetics of cyt b_5, two mutants of trypsin-solubilized bovine liver cytochrome b_5 have been prepared. Glu44, Glu56, and Asp60 were mutated into alanine in mutant I (E44A/E56A/D60A) while Glu44, Glu48, Glu56, and Asp60 were mutated into alanine in mutant II (E44A/E48A/E56A/D60A). The electron self-exchange rates of cyt b_5 mutant I and mutant II have been measured as a function of temperature and ionic strength using one-dimensional ^{1}H NMR saturation transfer. The dipole moments as well as the reorganization energies for both cyt b_5 mutant I and mutant II were calculated. The results indicate that the four acidic amino acid residues near the exposed heme edge, E44, E48, E56, and D60, play an important role in the electron self-exchange reaction of cyt b_5 mainly because of the electrostatic barrier they create. We can conclude that the electron self-exchange reactions of cyt b_5 are mainly dominated by electrostatic interaction.

Experimental Section

Materials. DNA restriction endonucleases, T4 DNA polymerase, ligase and kinase were purchased from Biolabs. [γ-^{32}P]dATP was obtained from Amersham. The pUC19 plasmid containing the synthetic microsomal cytochrome b_5 (82 residues in length) was a generous gift from Professor A. G. Mauk. The other bio-products were from Sigma. All chemicals were of reagent grade.

Protein Preparations. Unless specified otherwise, site-directed mutagenesis of the gene coding for the trypsin-solubilized cytochrome b_5 and other DNA manipulations were performed as described by Sambrook et al.[20] To achieve a high efficiency, we adopted some newly developed methods. We have used two types of synthesized and purified oligonucleotide mixture. Primers of the first type were same in the most deoxyribonucleotide sequence just except the triplet code site we wanted to generate multiple mutations, and in the second type, each primer of the mixture would introduce a different mutation to the cytochrome b_5 gene at a different site. In all cases, the same concentration of primers were used to obtain multiple mutants at a single site or at several different site of the cytochrome b_5 gene in just one process, separately.[21] The ratio of concentration during hybridization between the oligonucleotide mixture and the single-stranded DNA template containing cytochrome b_5 gene was 1:1 in most cases. By raising the relative quantity of the oligonucleotide mixture (to the above-mentioned second type only) in hybridization system, the probability of gaining mutants with multiple mutations at several site in one gene increased. Different mutants obtained in a single process were differentiated by dot blot hybridization screening and were picked out separately. All mutated cytochrome b_5 genes were sequenced by the dideoxynucleotide chain termination method[22] or/and by ABI PRISM model 377 automatic sequencer. All the mutated genes were ligated into *Eco*RI/*Hind*III cut pUC19 plasmid and then transformed into *Escherichia coli* host JM83. The expression of genes and the purification of proteins were accomplished according to Mauk's methods.[23]

NMR Sample Preparation. The NMR samples were prepared in sodium phosphate D_2O buffer ($\mu = 0.05$ M, pH = 7.0). Samples with ionic strength higher than 0.05 M were prepared by direct addition of NaCl to the NMR sample tube. The mutated ferricytochrome b_5 was reduced by the anaerobic addition of solid $Na_2S_2O_4$ directly into the NMR tube. Before and after the reduction operation, the samples were degassed and purged with nitrogen for several times. NMR measurements were performed immediately after preparation of the samples.[6]

Kinetic Measurements. All ^{1}H NMR spectra were acquired on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer system. The NMR probe temperature was calibrated to $\pm$0.1 °C with the use of a B-VT 2000 temperature unit. The carrier was centered on the residual water peak. Chemical shift values for all the resonances are referenced to internal 1,4-dioxane at 3.743 ppm. 1D saturation transfer experiments were used for calculation of the rate constants. The overall experiment was performed by applying a presaturation decoupler pulse either on-resonance for the saturation transfer spectrum, or off-resonance for the reference spectrum. On- and off-resonance frequencies were alternated every 16 scans. The difference spectra were generated by subtracting the reference spectra from the decoupler on-resonance spectra, in which the phenomenon of saturation transfer was most evident.[24−26]

The ratio of oxidized to reduced protein was determined from the integrated areas of the two forms of the protein in the NMR spectrum. Measurements of a single rate constant required about 2 h, during which time the ratio of Fe(II)/Fe(III) cytochrome b_5, as calculated from the peaks in the upfield region of ^{1}H NMR spectrum, was stable to better than $\pm$10%. The preirradiation of Leu25 methyl resonance of the oxidized protein decreases the intensity of the corresponding resonance of reduced protein. At equilibrium:[27,28]

$$I'_{\mathrm{Red}}/I_{\mathrm{Red}} = t_{\mathrm{Red}}/(t_{\mathrm{Red}} + T_{1\mathrm{Red}}) \quad (1)$$

where I'_{Red} and I_{Red} are the intensities of the Leu25 methyl resonances with or without presaturation of the resonance of the oxidized protein. t_{Red} is the lifetime of the protein in the reduced state and $T_{1\mathrm{Red}}$ is the longitudinal relaxation time of the reduced Leu25 methyl resonance measured in the absence of oxidized form. The electron self-exchange rate constants are calculated from the following equation:[27,28]

$$k_{\mathrm{ese}} = 1/t_{\mathrm{Red}}[\mathrm{Ox}] \quad (2)$$

The oxidized protein concentration can be expressed as:

$$[\mathrm{Ox}] = I_{\mathrm{ox}}/(I_{\mathrm{ox}} + I_{\mathrm{red}})[\mathrm{cyt}\ b_5] \quad (3)$$

I_{ox} and I_{red} are intensities of the oxidized and reduced Leu25 methyl resonance in the mixture of the oxidized and reduced proteins. [cyt b_5] is the sum of oxidized and reduced protein concentration.

The spin−lattice relaxation time T_1 of the reduced Leu25 methyl resonance was measured by the inversion recovery method. The ^{1}H NMR data were taken over the temperature range of 293−308 K.[29] The integrated values of signals in the NMR spectra were obtained by the aid of deconvolution method in UXNMR software. Trypsin-solubilized cyt b_5 is a mixture of two isomers and the two forms are in about a 10: 1 ratio.[30] The rate constants reported here were measured on the major isomer and it was assumed that the rates exhibited by the major and minor isomers were identical.

Results and Discussion

Kinetic and Thermodynamic Studies. NMR spectroscopy has provided an effective approach to analyze the electron self-

(18) Funk, W. D.; Lo, T. P.; Mauk, M. R.; Brayer, G. D.; MacGillivray, R. T. A.; Mauk, A. G. *Biochemistry* **1990**, *29*, 5500−5508.
(19) Lloyd, E.; Ferrer, J. C.; Funk, W. D.; Mauk, M. R.; Mauk, A. G. *Biochemistry* **1994**, *33*, 11432−11437.
(20) Sambrook, J.; Fristch, E. F.; Maniatis, T. In *Molecular Cloning*: *A Laboratory Manual*, 2nd edition; Cold Spring Harbor Laboratory Press: Plainview, NY, 1989.
(21) Yao, P.; Xie, Y.; Wang, Y. H.; Sun, Y. L.; Huang, Z. X.; Xiao, G. T.; Wang, S. D. *Protein Eng.* **1997**, *10*(5), 575−581.
(22) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci.* U.S.A., **1977**, *74*, 5463−5467.
(23) Funk, W. D.; Lo, T. P.; Mauk, M. R.; Brayer, G. D.; MacGillivray, R. T. A. M.; Mauk, A. G. *Biochemistry* **1990**, *29*, 5500−5508.
(24) Gupta, R. K.; Redfield, A. G. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 273−281.
(25) Shao, W.; Sun, H.; Yao, Y.; Tang, W. *Inorg. Chem.* **1995**, *34*, 680−687.
(26) Shao, W.; Yao, Y.; Liu, G.; Tang, W. *Inorg. Chem.* **1993**, *32*, 6112−6114.
(27) Concar, D. W.; Hill, H. A. O.; Moore, G. R.; Whitford, D.; Williams, R. J. P. *FEBS Lett.* **1986**, *205*, 15−19.
(28) Legrand, N.; Bondon, A.; Simonneaux, G.; Schejter, A. *Magn. Reson. Chem.* **1993**, *31*, S23−S26.
(29) Bendzko, P.; Pfeil, W. *Biochim. Biophys. Acta* **1983**, *742*, 669−676.
(30) McLachlan, S. J.; La Mar, G. N.; Burns, P. D.; Smith, K. M.; Langry, K. C. *Biochim. Biophys. Acta* **1986**, *874*, 274−284.

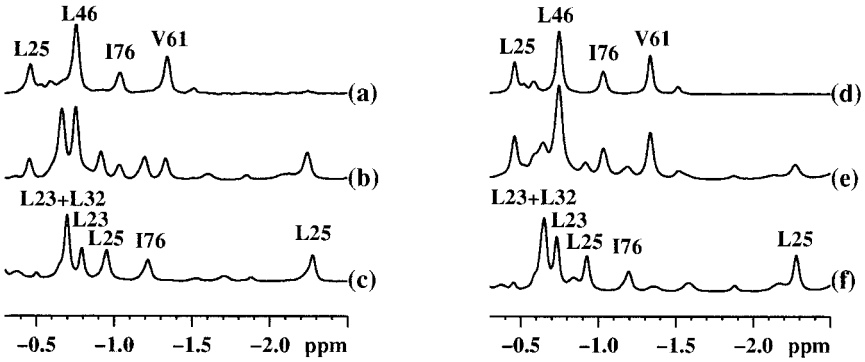

Figure 1. Upfield region of the NMR spectra of cyt b_5 mutants: (a−c) mutant I; (d−f) mutant II. (a, d) Reduced state; (b, e) mixture of reduced and oxidized states; (c, f) oxidized state.

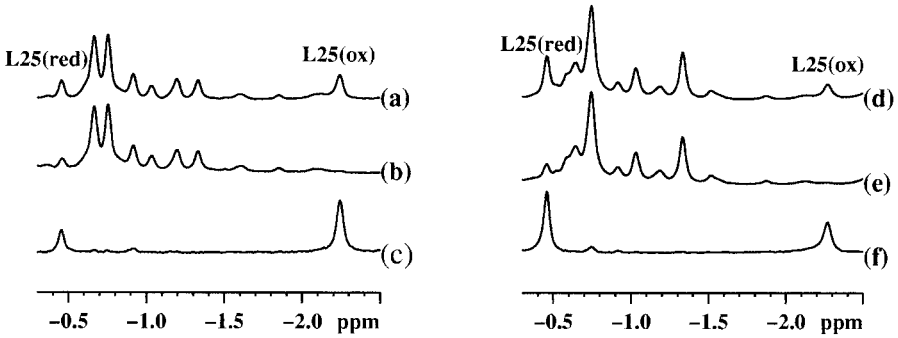

Figure 2. Saturation transfer difference spectra of a mixture of oxidized and reduced cyt b_5 mutants: (a−c) mutant I; (d−f) mutant II. (a, d) Reference spectra; (b, e) saturation transfer spectra upon irradiating at one of the oxidized L25 methyl resonances; (c, f) difference spectra.

Table 1. Kinetic Parameters for Electron Self-Exchange of Cyt b_5 Mutants

	mutant I[a]			mutant II[b]			wild type[c]
[cyt b_5] (mM)	0.50	0.40	1.5	0.60	0.40	1.5	1.0
ionic strength (M)	0.10	0.30	0.30	0.10	0.30	0.30	0.3
	Self-Exchange Rate Constants $\times 10^{-3}$ M^{-1} s^{-1}						
temp (°C)							
20	4.0 ± 0.4	7.7 ± 0.7	8.9 ± 0.7	8.4 ± 0.7	14 ± 1	14 ± 1	3.7
25	4.8 ± 0.4	11 ± 1	12 ± 1	9.5 ± 0.8	19 ± 1	21 ± 1	4.6
30	6.5 ± 0.5	12 ± 1	15 ± 1	13 ± 1	25 ± 2	26 ± 2	5.0
35	7.2 ± 0.7	15 ± 1	16 ± 1	17 ± 1	27 ± 2	29 ± 2	6.2
	Activation Parameters						
$\Delta H^{\ddagger}$, kcal mol^{-1}	6.8 ± 0.8	7.0 ± 0.9	6.6 ± 0.8	7.9 ± 0.9	7.9 ± 0.9	7.7 ± 0.9	5.8
$\Delta S^{\ddagger}$, eu	−19 ± 3	−17 ± 3	−18 ± 3	−14 ± 4	−13 ± 5	−13 ± 5	−22.4
$\Delta G^{\ddagger}$,[d] kcal mol^{-1}	12 ± 1	12 ± 1	12 ± 1	12 ± 2	12 ± 2	12 ± 2	12.5

[a] E44A/E56A/D60A mutant. [b] E44A/E48A/E56A/D60A mutant. [c] Data from ref 6. [d] 25 °C.

exchange reaction.[6−12] The self-exchange rates of cyt b_5 mutants are relatively slow on the NMR time scale. The upfield region of the NMR spectra of cyt b_5 mutant I and mutant II are shown in Figure 1, including the spectra of the oxidized form, the reduced form and the mixture of the oxidized and reduced forms. The full assignment for the resonances of cyt b_5 mutant II has been completed.[31] The chemical shifts of Leu25 methyl resonances of cyt b_5 mutants are very close to those of wild-type cyt b_5. Leu25 methyl resonances from both the reduced state and the oxidized state are shown in the upfield range of the NMR spectra in Figure 1. The saturation transfer spectra for the mixture of the two oxidation states of both cyt b_5 mutant I and mutant II are shown in Figure 2. Upon saturation of Leu25 methyl resonance in the oxidized protein, saturation transfer results in a smaller intensity of the same peak in the reduced protein. Given the integrated values of signals and the spin−lattice relaxation time T_1, the magnitudes of k_{ese} were calculated using eqs 1−3,[27,28] as described in Experimental Section. The electron self-exchange rate constant of cyt b_5 mutant I is (4.0 ± 0.4) × 10^3 M^{-1} s^{-1} at 20 °C with μ = 0.10 M and [cyt b_5] = 0.50 mM, while that of mutant II is (8.4 ± 0.7) × 10^3 M^{-1} s^{-1} at 20 °C with μ = 0.10 M and [cyt b_5] = 0.60 mM. The self-exchange rate constants of the two cyt b_5 mutants at four different temperatures and three combinations of protein concentration and ionic strength are listed in Table 1. The plots of ln(k/T) vs 1/T are shown in Figure 3. The intercept and slope, given by linear least-squares fits to the experimental data, yielded the entropy and enthalpy of activation using the Eyring equation. The Eyring parameters are also listed in Table 1.

From Table 1 and Figure 3, it is reflected that the kinetic data is hardly affected by different protein concentrations under the same temperature and ionic strength. At a given solution condition, the magnitude of k_{ese} for the three forms of protein descends in the following order: mutant II > mutant I > wild-type cyt b_5.[6] The mutation of Glu44, Glu56, and Asp60 to

(31) Lu, J.; Huang, Z.-X.; Tang, W.; et al. Unpublished material.

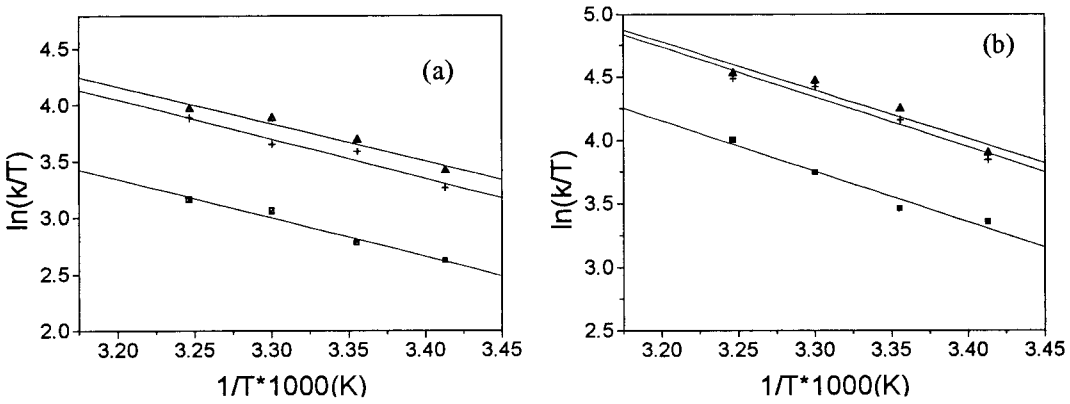

Figure 3. Eyring plots of cyt b_5 mutants electron self-exchange rate constants at various protein concentrations and ionic strengths. (a) Mutant I; (b) mutant II; (■) $\mu = 0.10$ M, [cyt b_5] = 0.50 mM; (+) $\mu = 0.30$ M, [cyt b_5] = 0.40 mM; (▲) $\mu = 0.30$ M, [cyt b_5] = 1.5 mM.

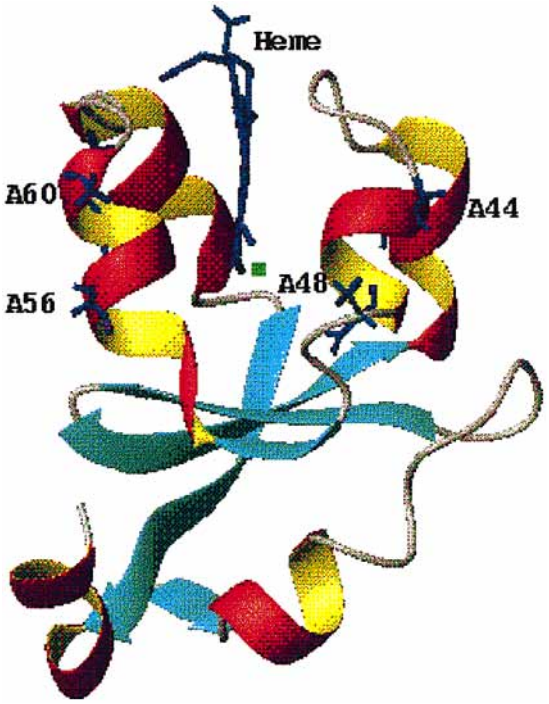

Figure 4. Ribbon diagram of cyt b_5 showing the residues 44, 48, 56, and 60, which are replaced by four alanines in wild-type cyt b_5 crystallographic structure (PDB entry pdb3b5c.ent). The figure was created with the program MOMOL.[33]

alanine increases the self-exchange rate constants significantly. Additional mutation of Glu48 by alanine brings even larger values for the self-exchange rate constants. So the Glu48 plays a much more important role in the cyt b_5 electron self-exchange reaction. It is well-known that cyt b_5 has a highly asymmetric distribution of charges.[32] Dixon et al. studied the electron self-exchange of cyt b_5 and proposed a heme edge-to-heme edge complex model, assuming that the electron self-exchange reactions occur at the partially exposed heme edge.[6] The E44, E48, E56, and D60 are all located near the exposed heme edge in wild-type cyt b_5 with their carboxylic groups fully extended into solution and completely exposed to solvent. As a result, these carboxylic groups provide a significant contribution to the electrostatic barrier, which is a disadvantage to the formation of the precursor complex for the self-exchange reaction of wild-type cyt b_5.[6] When these acidic residues are mutated into alanine (Figure 4), the electrostatic barrier between the two electron self-exchange reaction partners certainly decreased significantly with the elimination of the carboxylic groups. After mutation of these specific residues the increases of rate constant of electron self-exchange reaction of cyt b_5 is expected.

The temperature dependence of electron self-exchange reactions of cyt b_5 mutants are different from that of wild-type cyt b_5.[6] The values of $\Delta H^{\ddagger}$ became larger after mutation, while the absolute values of $\Delta S^{\ddagger}$ became smaller. The less negative values of $\Delta S^{\ddagger}$ are favorable for the electron self-exchange process. That is to say, the change in $\Delta S^{\ddagger}$ values should facilitate the electron self-exchange reactions of cyt b_5 mutants. On the other hand, it should be noted that the $\Delta H^{\ddagger}$ values are more positive for mutants than for wild-type cyt b_5.[6] The increase in $\Delta H^{\ddagger}$ values are unfavorable for the electron self-exchange process. In this study, the favorable effect on the self-exchange reaction brought about by the changes of $\Delta S^{\ddagger}$ is greater than the unfavorable effect brought about by the changes of $\Delta H^{\ddagger}$, leading to the increased values of k_{ese} in cyt b_5 mutants.

Electrostatic Studies. Information about the electrostatic interactions between two reactants can be obtained from the ionic strength dependence of electron self-exchange rate constants.[8,34,35] At pH 7.0 and 25 °C, the rate constant for electron self-exchange reaction of cyt b_5 mutant I is $(4.8 \pm 0.4) \times 10^3$ M^{-1} s^{-1} with $\mu = 0.10$ M. The k_{ese} values increase monotonically with increasing ionic strength as shown in the ionic strength dependence (Figure 5a). The cyt b_5 mutant II has similar ionic strength dependence of the self-exchange reaction shown in Figure 5b. The dipole moment can be obtained by a fit of the ionic strength dependence of electron self-exchange rate constants.[6,35−37] Van Leeuwen's approach[36] is used to treat the ionic strength dependence of self-exchange reactions. The approach presumed that electron transfer takes place on the partially exposed heme edge and it has proved to be effective at high ionic strength, which is needed for NMR studies. Equations are as follows:

$$\ln(k_{ese}/k_{inf}) = -\{Z_{ox}Z_{red} + (ZD)(1 + \kappa r) + (DD)(1 + kr)^2\}(q^2/4\pi\epsilon_0\epsilon k_B Tr)f(\kappa) \quad (4)$$

$$ZD = (Z_{ox}D'_{red} + Z_{red}D'_{ox})/qr \quad (5)$$

$$DD = D'_{ox}D'_{red}/(qr)^2 \quad (6)$$

$$f(\kappa) = (1 - \exp(-\kappa r))/\kappa r(1 + \kappa r/2) \quad (7)$$

where Z_{ox} and Z_{red} are the net charges of the oxidized and reduced protein; D'_{red} and D'_{ox} are the components of the dipole moments through the exposed heme edge; r is the sum of the

(32) Koppenol, W. H.; Margoliash, E. *J. Biol. Chem.* **1982**, *257*, 4426−4437.

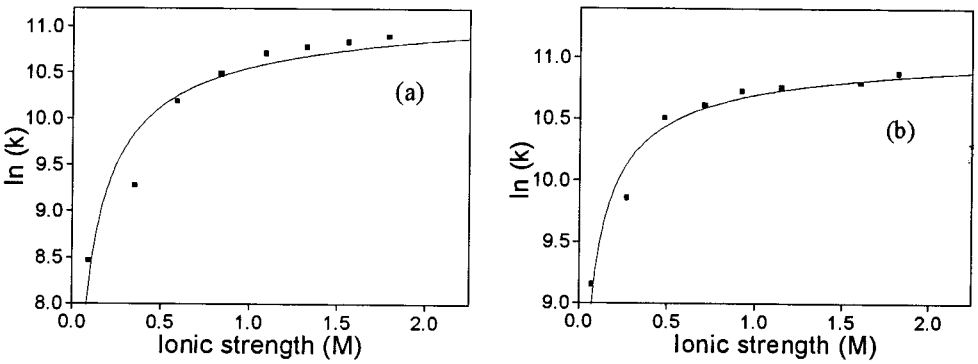

Figure 5. The ionic strength dependence of cyt b_5 mutants electron self-exchange rate constant (pH 7.0, 25 °C). The solid line is a best fit to the experimental data. (a) Mutant I: fitted values are $D'_{ox} = -220$ D, $D'_{red} = -254$ D, and $k_{inf} = 2.8 \times 10^5$ M^{-1} s^{-1} for $Z_{ox} = -6$ and $Z_{red} = -7$. (b) Mutant II: fitted values are $D'_{ox} = -134$ D, $D'_{red} = -159$ D, and $k_{inf} = 1.1 \times 10^5$ M^{-1} s^{-1} for $Z_{ox} = -5$ and $Z_{red} = -6$. The dipole moments are the component of the dipole moment through the exposed heme edge.

radii of the two electron-transfer partners ($r = 31.8$ Å); $\kappa = 0.329\mu^{1/2}$, and μ, ionic strength; k_{ese} is the rate constant at a given ionic strength, and k_{inf} is the rate constant at infinite ionic strength. Other abbreviations used are as follows: q, elementary charge in coulombs ($q = 1.6 \times 10^{-19}$ C); k_B, Boltzmann's constant (1.3807×10^{-23} J K^{-1}); ϵ_0, permeativity constant (8.85418×10^{-12}); ϵ, static dielectric constant (80). It is assumed that all arginine and lysine residues and the amino-terminus are protonated and that all carboxylic acids (including those of the heme and carboxyl-terminus) are dissociated; and histidines are in neutral form. Given $Z_{ox} = -6$ and $Z_{red} = -7$ for mutant *I*, $Z_{ox} = -5$ and $Z_{red} = -6$ for mutant II, a fit of the current data to these equations gives $D'_{ox} = -220$ D, $D'_{red} = -254$ D, and $k_{inf} = 2.8 \times 10^5$ M^{-1} s^{-1} for cyt b_5 mutant I, and $D'_{ox} = -134$ D, $D'_{red} = -159$ D, and $k_{inf} = 1.1 \times 10^5$ M^{-1} s^{-1} for cyt b_5 mutant II. For wild-type cyt b_5, the corresponding values are $D'_{ox} = -250$ D, $D'_{red} = -280$ D, and $k_{inf} = 3.7 \times 10^5$ M^{-1} s^{-1}.[6] It has been noticed that the fitted values of dipole moments are sensitive to the value of ionic strength of the solution.[8] In this work, the ionic strength is calculated on the basis of small ion concentrations plus the charge on the protein multiplied by the protein concentration.[6,8] The dipole moment of the protein decreased after mutation.[6] The consideration of the asymmetric charge distribution on the proteins[32] is very important in order to understand the changes in dipole moments of cyt b_5 mutants. After the mutation of E44, E56, and D60 by alanine, the charge distribution of cyt b_5 becomes less asymmetric. This is consistent with the smaller absolute values of dipole moments in mutant I than in wild-type cyt b_5. The absolute values of dipole moments of mutant II are even smaller than those of mutant I.

In addition, van Leeuwen's approach allows the calculation of an interaction energy, $w_r = -RT[\ln(k_{ese}/k_{inf})]$, for the two-electron self-exchange partners in a heme edge-to-heme edge geometry. The calculated values are 1.9 and 1.0 kcal mol^{-1} for cyt b_5 mutant I and mutant II, respectively. The corresponding value for wild-type cyt b_5 is 3.1 kcal mol^{-1} reported by Dixon et al.[6] The smaller the w_r value, the faster the electron self-exchange reaction. It is consistent with the results obtained under experimental conditions.

Reorganization Energy. The reorganization energy (λ), defined as the energy required to distort the nuclear configuration of product state into the geometry of the reactant state without electron transfer,[38] is a very important factor in determining the rate of electron self-exchange reaction. The reorganization energy can be calculated in many ways.[3,6,39] We used the approach developed by Dixon et al.[6] for calculating the reorganization energy of self-exchange reactions of the cyt b_5 mutants.

With use of Marcus formalism, the self-exchange rate constant can be expressed as[3,6]

$$k_{ese} = SK_a\nu_n\kappa_{el}\exp(-\Delta G_f^*/RT) \quad (8)$$

where S is the steric factor, K_a is the association constant for formation of the precursor state from the two separated electron self-exchange partners, ν_n is the nuclear frequency factor, κ_{el} is the probability of electron tunneling and ΔG_f^* is the free energy of activation. The association constant can be estimated by the following equation:[6]

$$K_a = 4\pi N r^2 \delta(r)\exp(-\omega_r/RT) \quad (9)$$

where N is Avogadro's number, r is the sum of the radii of the two proteins (31.8 Å),[6] the value of $\delta(r)$ (1.11 Å) is usually taken as β^{-1}, the distance at which the electron self-exchange rate constant decreases to 1/e of its value in the heme edge-to-heme edge complex,[6] and with the use of van Leeuwen formalism, $\exp(-w_r/RT) = k_{ese}/k_{inf}$.[12] Given these values, K_a is calculated to be 0.33 and 1.5 M^{-1} for cyt b_5 mutant I and mutant II, respectively. For wild-type cyt b_5, $K_a = 0.045$ M^{-1} as reported by Dixon et al.[6] The association constant for the precursor complex of cyt b_5 mutant I is more than seven times larger than that of wild-type cyt b_5. The association constant for the precursor complex of cyt b_5 mutant II is 4.5 times larger than that of cyt b_5 mutant I. It indicates that the reduction of surface charge by mutation of the charged residues to neutral residues is favorable for the formation of the precursor complex to a large extent. The value of steric factor S is estimated by Dixon et al. as 0.036.[6] $\nu_n k_{el}$ can be expressed as[3,6]

$$\nu_n k_{el} = 10^{13}\exp[-\beta(d - d_0)] \quad (10)$$

(33) Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graphics* **1996**, *14*, 51−55.
(34) Mauk, M. R.; Mauk, A. G.; Weber, P. C.; Matthew, J. B. *Biochemistry* **1986**, *25*, 7085−7091.
(35) Rush, J. D.; Lan, J.; Koppenol, W. H. *J. Am. Chem. Soc.* **1987**, *109*, 2679−2682.
(36) Van Leeuwen, J. W. *Biochim. Biophys. Acta* **1983**, *743*, 408−421.
(37) Rush, J. D.; Koppenol, W. H. *Biochim. Biophys. Acta* **1988**, *936*, 187−198.
(38) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796−802.
(39) Muegge, I.; Qi, P. X.; Wand, A. J.; Chu, Z. T.; Warshel, A. *J. Phys. Chem.* B **1997**, *101*, 825−836.

where d (7.5 Å) is estimated to be the closest heme-heme distance, $d_0 = 3$ Å, and $\beta = 0.9$ Å^{-1}.[6] The value for $\nu_n k_{el}$ is 1.74×10^{11} s^{-1}. The values of ΔG_f* are calculated to be 7.2 and 7.8 kcal mol^{-1} for cyt b_5 mutant I and mutant II, respectively. The corresponding value for wild-type cyt b_5 is 6.9 kcal mol^{-1}.[6] According to Marcus formalism,[3,6]

$$\Delta G_f^* = (\lambda/4)(1 + \Delta G_0'/\lambda)^2 \quad (11)$$

$$\Delta G_0' = \Delta G_0 + w_p - w_r \quad (12)$$

Here, ΔG_0 is the free energy change of the reaction, and w_p and w_r represent the work required to bring the reactants and products to separation achieved in the electron-transfer complex. For self-exchange reactions, $w_p = w_r$ and ΔG_0 is zero, so, $\lambda = 4\Delta G_f^*$. The calculated values of λ are 1.2 and 1.3 eV, for cyt b_5 mutant I and cyt b_5 mutant II respectively, compared with the value of 1.2 eV for wild-type cyt b_5.[6] It should be noted that the heme edge-to-heme edge distance for cyt b_5 mutants might decrease due to the reduced electrostatic repulsion because of the reduction of net charges in cyt b_5 mutants. However, even a decrease in the heme edge-to-heme edge distance of 2 Å would lead to only small change on the reorganization energy,[6] so it is acceptable to adapt the heme edge-to-heme edge distance of wild-type cyt b_5 in calculating the reorganization energies of cyt b_5 mutants.

The values of reorganization energies of mutants only change slightly compared with the λ value of wild-type cyt b_5, that is, the energies required for the oxidized state protein to assume the geometry of the reduced partner in mutants and in wild-type cyt b_5 are similar.[6] In addition, according to the structural similarity between wild-type cyt b_5 and cyt b_5 mutants,[31] the degree of the heme exposures of mutants and wild-type cyt b_5 are nearly the same. The different values of k_{ese} for wild-type cyt b_5 and mutants at a given ionic strength and temperature may be attributed to the electrostatic effect. The change in electrostatic effect becomes more favorable to the electron self-exchange process of cyt b_5 after mutation of the negatively charged amino acid residues by alanine. It could be concluded that mutation selectively alters the electrostatic interaction, which is thought to be involved in protein−protein recognition.[40,41] Removal of acidic residues near the exposed heme edge by site-directed mutagenesis facilitated the self-exchange reactions of cyt b_5 favorably under the experimental conditions. In other words, the current work supports the heme edge-to-heme edge model for cyt b_5 self-exchange complex.[6]

Acknowledgment. This project is supported by the National Natural Science Foundation of China and the State Key Laboratory of Coordination Chemistry of Nanjing University and the State Key Laboratory of Genetics of Fudan University.

IC990607K

(40) Qian, W.; Sun, Y. L.; Wang, Y. H.; Zhuang, J. H.; Xie, Y. *Biochemistry* **1998**, *37*, 14137−14150.
(41) Sun, Y. L.; Wang, Y. H.; Yan, M. M.; Sun, B. Y.; Xie, Y.; Huang, Z. X.; Jiang, S. K.; Wu, H. M. *J. Mol. Biol.* **1999**, *285*, 347−359.