Electrostatic effect on electron transfer between cytochrome b_5 and cytochrome c*

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Abstract The binding and electron transfer between wild type, E44A, E56A, E44/56A, E44/48/56A/D60A and F35Y variants of cytochrome b_5 and cytochrome c were studied. When mixed with cytochrome c, the cytochrome b_5 E44/48/56A/D60A did not show the typical UV-vis difference spectrum of absorption, indicating that the alteration of the surface electrostatic potential obviously influenced the spectrum. The electron transfer rates of wild type cytochrome b_5, its variants and cytochrome c at different temperature and ionic strength exhibited an order of F35Y > wild type > E56A > E44A > E44/48/56A/D60A. The enthalpy and entropy of the reaction did not change obviously, suggesting that the mutation did not significantly disturb the electron transfer conformation. The investigation of electron transfer rate constants at different ionic strength demonstrated that electrostatic interaction obviously affected the electron transfer process. The significant difference of Cyt b_5 F35Y and E44/48/56A/D60A from the wild type protein further confirmed the great importance of the electrostatic interaction in the protein electron transfer.

Keywords: cytochrome b_5, cytochrome c, site-directed mutagenesis, electron transfer, electrostatic interaction.

Electron transfer (ET) is a basic and crucial reaction in many complicated biological processes such as respiratory chain, photosynthesis and xenobiotic process. Cytochrome b_5 (Cyt b_5) and cytochrome c (Cyt c) are small proteins, and their structures have been well characterized both in reduced and oxidized forms, so that they were chosen as a useful model to investigate the fundamental mechanisms regarding interprotein ET[1].

At present, many experimental and theoretical studies have been carried out to determine the mechanism of the ET in the Cyt b_5/c system[1—7]. It is now generally accepted that several lysine residues of Cyt c and glutamate or aspartate residues of Cyt b_5 are involved in forming salt bridges in the ET[3,6], and the ET process is also influenced by the thermodynamic driving force and hydrophobic interaction[7]. The modeling study showed that Cyt b_5 and Cyt c formed a 1∶1 precursor complex by electrostatic interaction to transport electron[3,4]. Rodgers and Sliger[5] suggested that the electrostatic contribution in the Cyt b_5 and Cyt c interaction was only 14%, and other nonelectrostatic forces, such as hydrogen bond, hydrophobic interaction, van der Waals' interaction and so forth, were the major factors.

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In order to further elucidate the influence of the electrostatic interaction on the ET, site-directed mutagenesis was used to change the carboxyl amino acids (E44, E48, E56 and D60) on the surface of Cyt b_5 to alanine, and four variants of Cyt b_5, E44A, E56A, E44/56A and E44/48/56A/D60A, were obtained. According to the modeling study, the Cyt b_5 E44/48/56A/D60A mutant changed all the proposed negatively charged residues on the protein surface involved in potential salt linking with Cyt c into neutral alanine. Based on the previous reports[7], the binding and ET between Cyt b_5 E44/48/56A/D60A mutant and Cyt c are mainly concerned here. At the same time, the importance of the electrostatic interaction and of the driving force on the ET process are explored by comparing the results with those of the Cyt b_5 F35Y mutant.

1 Materials and methods

All chemical reagents are of analytic grade. Double-distilled water was used in the preparation of all the solutions. Methyl viologen and proflavine hemisulfate were obtained from Tokyo Chemical Industry Co. Ltd., Japan.

The construction of the recombinant bovine liver microsomal Cyt b_5 trypsin-solubilized fragment and its mutants, the expression of the constructs in *E. Coli*, and the proteins' isolation, purification were performed as previously reported[6]. Horse heart Cyt c (type VI) was purchased from Sigma Chemical Co. and purified according to the description[8]. The purified protein solution was desalted and concentrated by ultrafiltration (Amicon 8050, YM-3 membrane), and after lyophilized, the protein was stored at -20℃. The protein concentrations were determined spectrophotometrically based on $\varepsilon_{412.5} = 117\ 000\ (\text{mol/L}\cdot\text{cm})^{-1}$ for Cyt b_5[9] and $\varepsilon_{410} = 106\ 100\ (\text{mol/L}\cdot\text{cm})^{-1}$ for Cyt c[10].

1.1 Binding between Cyt b_5 E44/48/56A/D60A mutant and Cyt c

According to ref. [8], protein solutions were prepared by dissolving the lyophilized proteins in sodium phosphate buffer (pH = 7.0, I = 1 mmol/L). The difference spectra were obtained using a tandem mixing cell (path length 2×0.438 cm) and recorded on an HP8452A diode array spectrophotometer.

1.2 Electron transfer kinetics of wild type Cyt b_5 and its mutants with Cyt c

Rapid-mixing experiments were performed with an SF-61DX2 Double-Mixing Stopped-Flow spectrophotometer (Hi-Tech Limited). The temperature was maintained by a NESLAB RTE-5B circulating bath instrument with a precision of ± 0.2℃. The ionic strength was adjusted by addition of NaCl. The measurement and the deaerating method were described else where[6]. Under the pseudo-first-order conditions, the kinetics of oxidation of ferro-Cyt b_5 and its mutants by ferri-Cyt c were monitored at wavelength of 428 nm, which is an isosbestic point for ferri-/ferro-Cyt c, and rate constant was calculated. All measurements were repeated at least five times.

2 Results and discussion

The mechanism of electron transfer can probably be divided into two separate steps, the highly specific binding between redox protein partners and the actual electron transfer within the bound complex[1]. So here, the binding and ET process are studied.

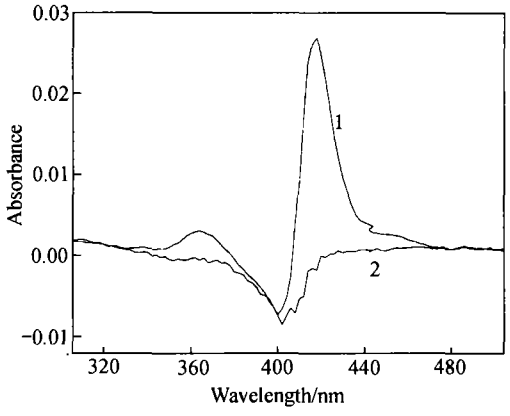

Fig. 1. Difference spectra of the reactions of wild type and E44/48/56A/D60A mutant Cyt b_5 with Cyt c. T = 25 ± 0.2℃, pH = 7.0, I = 1 mmol/L, concentrations of Cyt c and Cyt b_5 are both 6 μmol/L. Curve 1, wild type Cyt b_5 and Cyt c; curve 2, E44/48/56A/D60A mutant Cyt b_5 and Cyt c.

Figure 1 shows the difference spectra obtained from the reactions of wild type and E44/48/56A/D60A mutant of Cyt b_5 with Cyt c. It is interesting to note that E44/48/56A/D60A mutant exhibits no UV-vis absorbance difference in γ-band region. The previous studies demonstrated that the changes of the Soret absorption accompanying the protein complex formation are the result of the disturbance of the heme coordination environment, and the measurement was a classical method to examine the binding of the two hemoproteins. With the reduction of the charged residues on the surface of Cyt b_5, the amplitude of difference spectra decreased; however, the absorbent peaks were still visible[5,6]. The results from Cyt b_5 E44/48/56A/D60A mutant suggested that the binding of the two proteins was significantly altered. This might result from the substitution of the negatively charged residues by alanine on the surface, or more possibly, the binding geometry of the two proteins was changed in the ET modeling. Therefore, the electrostatic interaction is very important in steering the binding between Cyt b_5 and Cyt c.

Under the pseudo-first-order conditions, we determined the rate constants, enthalpies and entropies of the ET for the wild type Cyt b_5 or its mutants and Cyt c (table 1). The value of wild type Cyt b_5 is quite well in accord with those of Eltis et al.[2]. Table 1 shows that under the same experimental conditions, the order of rate constants for ET between surface mutants of Cyt b_5 and Cyt c is the following: wild type > E56A > E44A > E44/48/56A/D60A. It indicates that the ET efficiency drops with the decrease of the surface negatively charged residues. Because the reduction potential of the mutant protein did not alter significantly as compared with that of the wild type (table 1), the perturbation of the ET rate constant was mainly caused by the electrostatic interaction. As the rate constant of Cyt b_5 E44/48/56A/D60A variant dropped most obviously, it is probably caused by the drastic change of the precursor complex formation, considering its performance in binding with Cyt c.

Table 1 The reduction potentials of wild type Cyt b_5 and its mutants, and the kinetic and dynamic parameters of ET between Cyt b_5 variants and Cyt c[a)]

	WT	E56A	E44A	E44/56A	E44/48/56A/D60A	F35Y
E°/mV	4.5[6]	6.3[6]	6.0[6]	7.5[6]	15	-64[8]
$k_{12}/\times 10^7$ mol^{-1}s^{-1}	1.79	1.75	1.48	1.39	1.14	2.45
	(±0.22)	(±0.23)	(±0.19)	(±0.18)	(±0.14)	(±0.25)
$\Delta H^{\neq}$/kJ·mol^{-1}	31.3	30.9	26.7	32.8	32.5	31.8
$\Delta S^{\neq}$/J·mol^{-1}·K^{-1}	-1.33	-3.50	-18.8	1.00	-1.00	3.30

a) I = 350 mmol/L, pH = 7.0, T = 25.0 ± 0.2℃. The values in parenthese are 99 % confidence interval.

The observation on the ET of Cyt b_5 F35Y variant showed that its ET rate was much faster than that of the wild type Cyt b_5 (table 1). So far, it is the fastest ET rate among Cyt b_5 variants as far as we know. The F35 residue of Cyt b_5 is located in the hydrophobic patch of the heme hyprophobic pocket[1], far from the proposed interface of the protein complex, and the F35Y mutation does not disturb the overall charge of the protein. Thus, it can be concluded that the F35Y variant dose not affect the electrostatic interaction of Cyt b_5/c complex. However, replacing F35 by tyrosine disturbed the coordinating environment of the central iron ion which would account more than 66 mV[8] for the drop of the protein reduction potential, and the obvious increase in the driving force of ET between Cyt b_5 and Cyt c. Since the ET efficiency of F35Y mutant is higher than that of the wild type, it clearly shows that the driving force affects the ET at the same time.

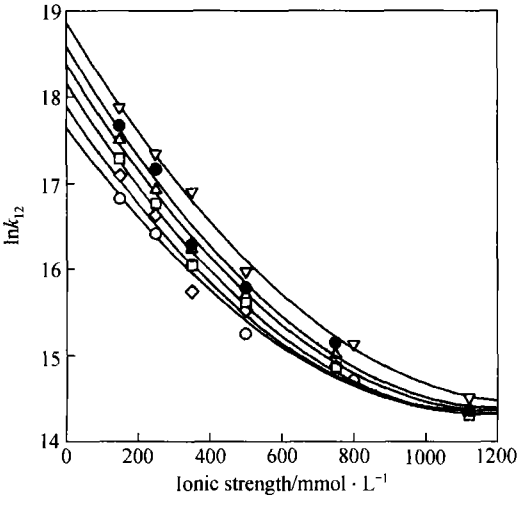

Fig. 2. Ionic strength dependence of the ln k_{12} of wild type Cyt b_5 and its variants on Cyt c ($T = 15.0 \pm 0.2$℃, pH = 7.0). wild type (●), E44A (□), E56A (△), E44/56A (▽), E44/48/56A/D60A (◇), and F35Y (□).

The calculated enthalpy and entropy are also listed in table 1. The results demonstrate that except for E44A mutant the values of all protein variants concerned did not change too much as compared with that of the wild type. Eltis et al.[2] also had the similar observation in DME-Cyt b_5 (esterified the heme propionates of Cyt b_5) and Cyt c system. This indicates that mutation does not significantly influence the conformation of ET between the two proteins.

Based on the previous studies, we changed the ionic strength of the reaction system in order to further elucidate the electrostatic effect on ET between Cyt b_5 and Cyt c. Using the SF-61DX2 double-mixing stopped-flow spectrometer with the detection limit of 1 ms, the rate constants were obtained in a relatively wide range of ionic strength (from 150 to 1120 mmol/L), and the results are shown in fig. 2.

At 150 mmol/L ionic strength, the ET rate was the fastest, and the differences among the rate constants of the protein variants were the most remarkable. With the increased ionic strength, the rate constants were declined and the rate differences among them were narrowed as well. Because the electrostatic interaction was gradually eliminated by the increased ionic strength, this suggests that ET process is affected by the electrostatic interaction. While the ionic strength was raised up to 1120 mmol/L, the rate constants of all derivatives, except for F35Y mutant, were essentially identical within the experimental measuring limits. It is interesting to find that the rate constant of Cyt b_5 E44/48/56A/D60A mutant is also equal to that of the wild type protein, suggesting that the electrostatic effect caused by complementary charges is eliminated almost completely at this ionic strength. As the

1) Yao, P., Huang, Z. X., Jian, W. et al., The X-ray and NMR structural study of cytochrome b_5 F35Y (to be published).

driving force of Cyt b_5 F35Y mutant is higher than any other mutant proteins, it exhibits higher ET rates at any ionic strength, especially at high ionic strength. The above results once again demonstrate that the differences of ET rates among different mutated Cyt b_5 molecules are caused by different electrostatic interactions.

By Tollin's expression[2], the local charges of the ET interface were calculated by the curve fitting of the plot of ET rate constant versus the ionic strength (table 2). The calculated value of charges on wild type Cyt b_5 was consistent with the values suggested by previous modeling and experimental researches[2—4]. Table 2 shows that the local charges of the ET interface decrease with the dropping of surface charges, and the E44/48/56A/D60A mutant drops the most significantly. It strongly suggests that the declining of ET rate constant by replacing the surface charges is caused by the decrease of electrostatic interaction. Compared to the above ET rate constant, the value of F35Y mutant suggests that its rate constant enhancing is attributed to the increase of driving force. In conclusion, comparing the ET of Cyt b_5 F35Y with that of the wild type, and considering the great difference between the E44/48/56A/D60A muant and the wild type on both binding and ET reaction, it clearly demonstrates that the electrostatic interaction and the thermodynamic driving force do affect the ET process between Cyt b_5 and Cyt c at the same time.

Table 2 The fitted local charges of the interface in Cyt b_5 and Cyt c ET[a)]

	WT	E56A	E44A	E44/56A	E44/48/56A/D60A	F35Y
Z	−4.5	−4.5	−4.3	−4.2	−4.0	−4.6

a) The fitting expression and the fitting parameters are from reference [2].

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