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Role of Carboxyl Residues Surrounding Heme of Human Cytochrome b_5 in the Electrostatic Interaction with NADH-Cytochrome b_5 Reductase

Masaya Kawano, Komei Shirabe,[1] Takushi Nagai, and Masazumi Takeshita
Department of Biochemistry, Oita Medical University, Hasama-machi, Oita 879-5593, Japan

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To identify the cytochrome b_5 residues responsible for the electrostatic interaction with NADH-cytochrome b_5 reductase (b_5R), we prepared and characterized the cytochrome b_5 mutants in which Glu41, Glu42, Glu63, Asp70, and Glu73 were replaced by Ala, utilizing site-directed mutagenesis and the expression system for cytochrome b_5 in *Escherichia coli.* Apparent Km values of the wild type b_5R for Glu42Ala cytochrome b_5 and Asp70Ala cytochrome b_5 were approximately three-fold and six-fold higher than that for the wild type cytochrome b_5, respectively, while the kcat values for those mutants were not remarkably affected. In contrast, Glu41Ala, Glu63Ala, and Glu73Ala cytochrome b_5 showed almost the same kinetic properties as the wild type cytochrome b_5. Furthermore, kinetic studies on combinations of the cytochrome b_5 and b_5R mutants suggested the interaction between Glu42 and Asp70 of cytochrome b_5 and Lys125 and Lys41 of b_5R, respectively, in the reaction. © 1998 Academic Press

Cytochrome b_5 is a heme protein which receives one electron from b_5R (1) or NADPH-cytochrome P450 reductase (2) and transfers it to other proteins, such as β-ketoacyl CoA reductase (3), 4-methylsterol oxidase (4), CMP-N-acetylneuraminic acid hydroxylase (5), cobalamine reductase (6), and cytochrome P450 (7). In human erythrocyte, soluble form cytochrome b_5 lacking a membrane binding fragment plays a role in the reduction of methemoglobin (1).

The electrostatic interaction between cytochrome b_5 and cytochrome c, an artificial electron donor and acceptor, has been extensively analyzed as a model system for the study of electron transfer mechanism (8). The X-ray crystallographic studies by Mathews *et al.* (9) revealed that 10 carboxyl residues outlined heme of bovine cytochrome b_5 serve a role in the interaction with other proteins. The model-building studies of cytochrome b_5-cytochrome c complex suggested that Glu47 (Glu43 in bovine; in the present study the numbering of the amino acid residues is based on the sequence of human cytochrome b_5), Glu48, Glu52, Asp64, and heme propionate of bovine cytochrome b_5 interact with lysyl residues of cytochrome c (10, 11). The electrostatic interaction of Glu48, Glu52, and Asp64 of cytochrome b_5 with lysyl residues of cytochrome c was clearly demonstrated by site-directed mutagenesis (12).

For the interaction between cytochrome b_5 and b_5R, authentic electron acceptor and donor, Strittmatter *et al.* demonstrated possible interactions of Glu47 and/or Glu48, Glu52 and/or Glu60, and heme propionate of bovine cytochrome b_5 with Lys41, Lys125, and Lys163 of b_5R by the chemical modification (13-15). The participation of the above listed Lys in the interaction was confirmed by site-directed mutagenesis by Strittmatter *et al.* (15) and Shirabe *et al.* (16). The replacement of all of Glu47, Glu48, Glu52, Glu60, and Asp64 of cytochrome b_5 by Ala, however, has no effect on the Km value of the reaction with b_5R (16). These results raised a possibility that cytochrome b_5 uses different set of acidic residues for the interaction with cytochrome c (electron acceptor) and with b_5R (electron donor).

In the present study in order to identify the cytochrome b_5 residues responsible for the electrostatic interaction with b_5R, we prepared and characterized the cytochrome b_5 mutants in which Glu41, Glu42, Glu63, Asp70, and Glu73 were replaced by Ala.

MATERIALS AND METHODS

Materials. Restriction enzymes and T4 polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan). *In vitro* oligonucleotide directed mutagenesis system was the product of Amersham (UK). Oligonucleotides for mutagenesis were the products of Sawady Co. (Tokyo, Japan). DEAE-Toyopearl 650 used for protein purification was the product of Tosoh (Tokyo, Japan). Sephacryl S-100 and RESOURCE Q were purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

[1] To whom correspondence should be addressed. Fax: 81-975-49-6302. E-mail: shirabe@oita-med.ac.jp.

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Site-directed mutagenesis and construction of the mutant cytochrome b_5 expression plasmid. The human erythrocyte cytochrome b_5 cDNA fragment cloned into the HindIII-EcoRI site of M13mp19 was obtained as described previously (17, 18). Site-directed mutagenesis was performed by the method of Taylor, using the Amersham mutagenesis system (19). Sense primers were designed as follows (the changed nucleotides were underlined): for Glu41Ala, the primer was 5′-AAATTTCTGGCAGAGCATCCT-3′; for Glu42Ala, the primer was 5′-TTTCTGGAAGCGCATCCTGGT-3′; for Glu63Ala, the primer was 5′-GAGAACTTTGCGGATGTCGGG-3′; for Asp70Ala, the primer was 5′-CACTCTACAGCTGCCAGGGAA-3′; for Glu73Ala, the primer was 5′-GATGCCAGGGCAATGTCCAAA-3′. M13 single strand DNA was prepared from five independent plaques after the transfection with the mutagenized phage DNA. Mutant clones were selected by dideoxy chain termination method (20). Each mutated HindIII-EcoRI fragment was excised from replicative form of the mutant M13 phage DNA and recloned into the same site of pKK223-3 (Pharmacia, Uppsala, Sweden). Each cytochrome b_5 mutant was overexpressed in *E. coli* RB791 as described previously (21). After overexpression, the wild type and cytochrome b_5 mutants were purified as described previously (21) and then purified further by the fast protein liquid chromatography on RESOURCE Q column to apparently homogeneity as judged by electrophoresis on polyacrylamide gel (15.0%) in the presence of SDS (22). The concentration of the cytochrome b_5 was determined from the reduced-oxidized absorbance difference at 424 nm using an extinction of 124 $mM^{-1} \cdot cm^{-1}$ (23).

Assay of enzyme activity. The human erythrocyte wild type, Lys41Ala, Lys125Ala, and Lys163Ala b_5R were prepared as described previously (21, 24). Enzyme activity was assayed by following the increase in absorbance at 424 nm (124 $mM^{-1} \cdot cm^{-1}$) caused by the reduction of cytochrome b_5 with excess NADH (100 μM) as described previously (21, 23). For the high concentration of cytochrome b_5 (more than 24 μM), increase at another absorption maximum [556 nm (19.3 $mM^{-1} \cdot cm^{-1}$)] of cytochrome b_5 was measured (23). The apparent Km for cytochrome b_5 and kcat values were determined by double reciprocal plots of initial velocity at various concentration of cytochrome b_5 as described previously (21, 24).

Graphic presentation of cytochrome b_5. Coordinates for bovine liver cytochrome b_5 (1CYO) was obtained from Brookhaven Protein Data Bank (25). For graphic model of cytochrome b_5, we utilized the computer graphic program developed by Fujitsu Kyushu System Engineering LTD [Protein Adviser (version 3.0), Fukuoka, Japan].

RESULTS

Preparation of cytochrome b_5 mutants. We prepared and characterized the cytochrome b_5 mutants in which one of Glu41, Glu42, Glu63, Asp70, and Glu73 were replaced by Ala, utilizing site-directed mutagenesis and expression system in *E. coli.* From 5 to 25 mg of the purified cytochrome b_5 mutants were obtained from 1 liter of *E. coli* culture. In the examination by SDS-polyacrylamide gel electrophoresis (15.0%), the migration of the cytochrome b_5 mutants was almost similar to that of the wild type cytochrome b_5. The molecular weight of the cytochrome b_5 mutants was calculated approximately 11,000 as previously described (data not shown) (23). Thermostabilities of the wild type and cytochrome b_5 mutants were examined by incubation for 10 minutes at 50°C. The activities of the wild type cytochrome b_5 and all mutants were almost unchanged, indicating that these mutants are as stable as the wild type (data not shown).

TABLE 1

Kinetic Properties of Wild-Type b_5R Using Human Erythrocyte Soluble Form Cytochrome b_5

Cytochrome b_5	k_{cat} (s^{-1})	Km (μM)	k_{cat}/Km $s^{-1} \cdot M^{-1}$ (mean)
Wild-type	600 ± 88	8.4 ± 2.0	7.1×10^7
Glu 41 Ala	591 ± 117	10.2 ± 3.1	5.8×10^7
Glu 42 Ala	617 ± 82	25.8 ± 5.1	2.4×10^7
Glu 63 Ala	625 ± 71	8.1 ± 1.6	7.7×10^7
Asp 70 Ala	413 ± 57	54.1 ± 12.6	7.6×10^6
Glu 73 Ala	642 ± 86	15.1 ± 3.0	4.2×10^7

Note. Each value represents the mean ± S.D. of the four independent experiments.

Kinetic properties of cytochrome b_5 mutants. The kinetic properties of various cytochrome b_5 mutants with the wild type b_5R were summarized in Table 1. Apparent Km values for Glu42Ala and Asp70Ala cytochrome b_5 were elevated to 25.8 ± 5.1 μM and 54.1 ± 12.6 μM which is approximately three-fold and six-fold higher than that for the wild type cytochrome b_5, respectively. On the other hand, Km values for Glu41Ala, Glu63Ala, and Glu73Ala cytochrome b_5 were not significantly changed. These results suggest that Glu42 and Asp70 interact with lysyl residues of b_5R.

Kinetic study using combinations of cytochrome b_5 and b_5R mutants. Lys41, Lys125, and Lys163 of b_5R were clearly shown to interact with acidic residues of cytochrome b_5 (14-16). Km values of Lys41Ala, Lys125Ala, and Lys163Ala mutants for cytochrome b_5 were elevated to 6.3-, 5.3-, and 5.7-fold of that of the wild type b5R. Increase in Km values might be due to loss of ionic interactions with negative residues, Glu42, Asp70, and possibly heme propionate of cytochrome b_5. There should be, therefore, at least three ionic bonds in the interaction. To identify pairs of amino acid residues of cytochrome b_5 and b5R which participate in the interaction we performed steady state kinetic analysis on combinations of the cytochrome b_5 and b5R mutants as shown in Table 2. kcat values of Lys125Ala b5R was dramatically reduced when the activity was measured with Asp70Ala cytochrome b_5 mutant, comparing with the values with the wild type and Glu42Ala cytochrome b_5. This is because two ionic bonds might be lost when Asp70Ala was used as a reaction partner, while only one was lost when the activity was measured with the wild type and Glu42Ala cytochrome b_5. Km value of Lys125Ala b5R was partially restored from 36.2 μM to 21.2 μM when Glu42Ala cytochrome b_5 was used as a substrate. These results suggest that Lys125 of b5R interact with Glu42 of cytochrome b_5. On the other hand in the case of Lys41Ala b5R mutant, Km value was restored from 42.3 μM to 12 μM when the value was measured with Asp70Ala instead of the wild type

TABLE 2

Kinetic Properties of b_5R Mutants Lys41Ala and Lys125Ala with Cytochrome b_5 Mutants Asp70Ala and Glu42Ala

	b_5R mutants					
	Lys41Ala			Lys125Ala		
Cytochrome b_5	k_{cat} (s^{-1})	Km (μM)	k_{cat}/Km $s^{-1} \cdot M^{-1}$ (mean)	k_{cat} (s^{-1})	Km (μM)	k_{cat}/Km $s^{-1} \cdot M^{-1}$ (mean)
Wild type[a]	125 ± 15	42.3 ± 3.2	2.9×10^6	472 ± 67	36.2 ± 4.3	1.2×10^7
Glu42Ala	18 ± 2	50.0 ± 8.5	3.6×10^5	258 ± 18	21.1 ± 2.5	1.2×10^7
Asp70Ala	30 ± 3	12.0 ± 2.4	2.5×10^6	27 ± 2	80.2 ± 12.7	3.3×10^5

Note. Each value represents the mean ± S.D. of the four independent experiments.
[a] Data are from Shirabe *et al.* (16).

cytochrome b_5, suggesting that Asp70 of cytochrome b_5 interact with Lys41 of b5R.

DISCUSSION

In the present study, we have identified Glu42 and Asp70 as negative residues which interact with Lys125 and Lys41 of b_5R, respectively.

The cytochrome b_5 is composed of six a-helices, five b-strands arranged in a b-sheet and several b-turns (26). There exist ten negative residues in the heme binding region of cytochrome b_5 (Figs. 1A and 1B). Glu47, Glu48, Glu52, Glu60, and Asp64 of cytochrome b_5 have been shown to participate in the interaction with electron acceptors, cytochrome c (10-12), cytochrome P450 (27, 28), and methemoglobin (29). Although the same set of residues was suggested to play roles in the interaction with b_5R by chemical modification experiments (14,15), the previous study by ourselves revealed that the replacement of all of Glu47, Glu48, Glu52, Glu60, and Asp64 of cytochrome b_5 by Ala has no effect on the Km value of the reaction with b_5R (16). These results prompted us to analyze the effect of mutagenesis of another set of negative residues, Glu41, Glu42, Glu63, Asp70, and Glu73. Thus all of ten acidic residues surrounding heme moiety were examined in this and previous studies by replacing by Ala, utilizing site-directed mutagenesis. Of ten residues, only the replacement of Glu42 and Asp70 decreased Km values of cytochrome b_5 for b_5R. Almost the same thermostabilities and CD spectra of cytochrome b_5 mutants as the wild type cytochrome b_5 (data not shown) suggest that there is no gross structural changes in these mutants. We concluded, therefore, that Glu42 and Asp70 might interact with lysyl residues of b_5R.

In the negative residues of cytochrome b_5 that interact with electron acceptors, Glu47, Glu48, and Glu52 are located in helix formed by residues 46-53, while Glu60 and Asp 64 exist in helix formed by residues 59-66 (Fig. 1B). On the other hand, Glu42 and Asp70, which were shown in this study to interact with b_5R, an electron donor, are located in helix formed by residues 37-42 and helix formed by residues 68-78, respectively (Fig. 1B). These results indicate that cytochrome

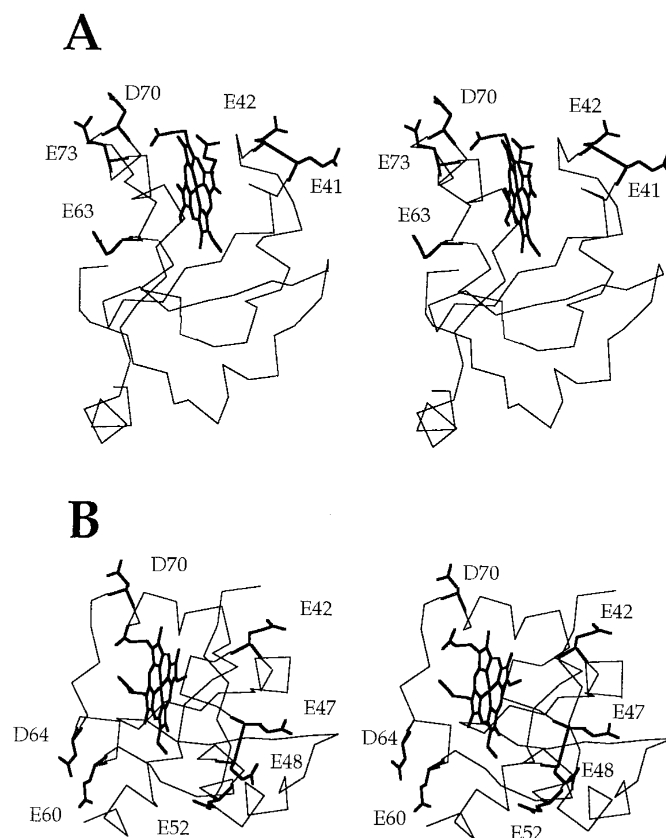

FIG. 1. Stereoscopic model of bovine cytochrome b_5. (A) The side chains of Glu41, Glu42, Glu63, Asp70, and Glu73 that are mutagenized in this study are shown. The numberings of these residues are taken from the sequence of human erythrocyte b_5. (B) Glu42 in helix formed by residues 37-42 and Asp70 in helix formed by residues 68-78 are shown to interact with b_5R in the present study. Glu47, Glu48, Glu52, Glu60, and Asp64 are the residues that interact with cytochrome c (10-12), cytochrome P450 (27, 28), and methemoglobin (29).

b_5 interacts with electron donor on one face and with electron acceptor on the other.

To identify the negative residues of cytochrome b_5 paired with Lys41, Lys125, and Lys163, kinetic studies were carried out for combinations of cytochrome b_5 and b_5R mutants. kcat/Km value of Lys41Ala b5R for Asp70Ala cytochrome b_5 mutant was the same level as for the wild type cytochrome b_5 in contrast to the decreased value for Glu42Ala cytochrome b_5 mutant (Tables 1 and 2). These results may indicate that loss of single ionic bond occurred in the reactions of Lys41Ala b5R versus the wild type cytochrome b_5 and Lys41Ala b5R versus Asp70Ala cytochrome b_5, while two ionic bonds were impaired in the reaction between Lys41Ala b5R and Glu42Ala cytochrome b_5. Thus Lys41 of b5R may interact with Asp70 of cytochrome b_5. In the case of Lys125Ala b5R, kcat/Km values for Glu42Ala cytochrome b_5 was the same as for the wild type, whereas the value for Asp70Ala cytochrome b_5 was one order lower. Thus kinetic studies of combinations of b_5R and cytochrome b_5 mutants suggested interactions of Glu42 of cytochrome b_5 with Lys125 of b_5R and Asp70 of cytochrome b_5 with Lys41 of b_5R.

These studies will lead us to modify the recently reported docking model for the complex of bovine cytochrome b_5 and porcine NADH-cytochrome b_5 reductase by Nishida (30) which include Glu48 of cytochrome b_5 (Glu44 in bovine) as a candidate residue for the interaction.

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