Probing the Role of Lysines and Arginines in the Catalytic Function of Cytochrome P450d by Site-directed Mutagenesis

INTERACTION WITH NADPH-CYTOCHROME P450 REDUCTASE*

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To identify amino acids of cytochrome P450d (P450d) which participate in the interaction with NADPH-cytochrome P450 reductase, we changed conserved ionic amino acids of P450d to others by site-directed mutagenesis. Turnover numbers (0.032–0.008 min⁻¹) of purified mutants Lys¹³'-Glu, Lys⁴⁸⁸'-Glu, Lys⁴₉⁹'-Glu, Lys⁴₉⁹'-Glu, and Lys⁶₄₃'-Glu toward 7-ethylcoumarin were much lower than that (0.380 min⁻¹) of the wild type at 25 °C. Reduction rates (less than 0.054 s⁻¹) of the heme of all mutants (0.1 μM) in the presence of NADPH and the reductase (0.3 μM) were much lower than that (5.9 s⁻¹) of the wild type. Furthermore, a turnover number (0.042 min⁻¹) of a microsomal triple mutant (Arg⁴₉⁵'-Leu + Arg⁴₉⁵'-Leu + Arg⁴₉⁵'-Leu) of a conserved Arg cluster was much lower than that (0.67k min⁻¹) of the wild type at 37 °C. Thus, we suggest that seven amino acids out of those mutated Lys and Arg residues of P450d are very important in the ionic interaction with the reductase and/or in orientating appropriate geometry for electron transfer on the interfacial surface between the two proteins.

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The abbreviations used are: P450, cytochrome P450; P450d, rat liver cytochrome P450d, which corresponds to P450IA2; P450rem, cytochrome P450 purified from Pseudomonas putida grown in the presence of camphor, which corresponds to P450C1; 7-ethylcoumarin, 7-ethyl-2H-1-benzopyran-2-one; acetalenilde, N-phenylacetamide; DLPC, dilauroyl-L-α-phosphatidylcholine.

For monooxidation reactions catalyzed by microsomal cytochrome P450 (P450),¹ two electrons must be supplied from NADPH to microsomal cytochrome P450 reductase in addition to molecular oxygen and organic substrates (1–4). Interactions of microsomal P450 with the reductase have been extensively studied in relationship with lipids (5–7), detergents (8–10), substrates (11), NADPH (12), and spin states (11). Electrostatic interactions between microsomal P450s with P450 reductase have been suggested (13–15).

For catalytic activities of bacterial P450rem, on the other hand, a non-heme iron protein, putidaredoxin, and a flavoprotein, NADPH-putidaredoxin reductase, are necessary as electron mediators from NADPH to P450rem. Putidaredoxin interacts directly with P450rem as a mobile shuttle between P450rem and putidaredoxin reductase (16). On the basis of the x-ray crystal structure of P450rem, specific amino acids such as Arg⁷⁷, Argⁱ³², Lys⁴⁴⁴, and Arg⁴⁶⁶ have been proposed for the surface residue of P450rem involved in the interaction with putidaredoxin (17, 18). In a similar way, mitochondrial P450s such as P450c₁ require a non-heme iron protein, adrenodoxin, and a flavoprotein, NADPH-adrenodoxin reductase, as electron mediators for catalytic activities (Ref. 19 and references cited therein). Adrenodoxin interacts with P450c₁ as a shuttle between P450c₁ and adrenodoxin reductase (19). Lys⁴⁸⁷ or Lys⁵⁸¹ (or Lys⁴⁵⁷) of P450c₁ has been suggested to be involved in the electrostatic interactions with adrenodoxin (20, 21).

NADPH-cytochrome P450 reductase is a flavoprotein and works as only one electron mediator to microsomal P450s from NADPH. Taking in consideration the above mentioned findings, it seemed very likely that ionic amino acids such as Lys and Arg on the protein surface of microsomal P450s directly interact with the reductase. Thus we made thirteen mutants of conserved Lys and Arg of P450d in the present study. Mutated positions of Lys and Arg of P450d and corresponding sequences of other P450s are shown in Fig. 1 (22, 23). Arg⁴⁵⁷-Leu and Lys⁶₄₃'-Glu mutants were obtained in our previous study (24). We obtained turnover numbers of mutants, dissociation constants of the reductase from mutants, and rates of the reduction of the heme of mutants by NADPH in the presence of the excess reductase. From those findings, we strongly suggest that seven amino acids out of those mutated Lys and Arg residues of P450d are very important in the ionic interaction with the reductase and/or in orientating the best geometry of the two proteins for the electron transfer.

**EXPERIMENTAL PROCEDURES**

Expression of P450d mutants in yeast was done as described previously (24–28). Site-directed mutageneses were done by using an in vitro mutagenesis kit of Amersham (United Kingdom) as previously described (24, 26). Mutation was confirmed by determination of nucleotide sequences by a Sequenase™ DNA sequencing kit of U. S. Biochemical Corp. We strictly checked every mutant for whether a mutation(s) at another position(s) had occurred.

Preparations of yeast microsomes and purification of P450d mutants were done as previously described (24–28). All mutants were purified as a high spin form, and most of them were stable both for oxidized and reduced forms. It should be noted, however, that the Lys⁴⁴⁴'-Glu and Lys⁴₉⁹'-Glu mutants were unstable at 37 °C in terms of the Soret absorption spectrum of the CO-reduced form. It was confirmed spectrometrically that these mutants are stable at 25 °C. Catalytic activities of microsomal and purified mutants were obtained as previously described (24, 28). Concentration of P450d mutants was obtained from molar absorptivity, 1.09 × 10⁴ M⁻¹ cm⁻¹ at 393 nm of the high spin oxidized form or 1.20 × 10⁴ M⁻¹ cm⁻¹ at 447 nm of the CO-reduced form. Concentration of the reductase was determined spectrometrically as previously described (6).
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Dissociation constants ($K_d$) of the reductase from the mutants were obtained from difference absorption spectra of the Soret region on a Jasco Uvidec-510 recording spectrometer. Rate constants of the reductase of the heme of the mutants were obtained on a stopped flow spectrometer (Union Rapid Reaction Analyzer RA-601) equipped with micromixing cells and directly connected with a NEC PC-9901VM personal computer.

RESULTS

Table I shows turnover numbers of microsomal ionic P450, mutants toward 7-ethoxycoumarin obtained at 37°C. Turnover numbers (0.046-0.110 min⁻¹) of Lys mutants at 94, 99, and 105 in the amino-terminal region were much lower than that of the wild type. Turnover numbers (0.225-0.620 min⁻¹) of single mutants of the "Arg cluster," Arg¹³⁵, Arg¹³⁶, Arg¹³⁷, were not much lower than that (0.674 min⁻¹) of the wild type, while that (0.042 min⁻¹) of the triple mutant of the Arg cluster was much lower than that of the wild type. Both Arg²⁷⁶-Leu and Arg²⁷⁹-Trp mutants did not bind the heme in the active site of this enzyme in terms of optical absorption spectroscopy. Mutant Lys⁶⁰¹-Glu had sufficient catalytic activities. Mutants Lys⁴⁴⁰-Glu, Lys⁴⁶⁰-Glu, Arg⁶⁵⁵-Glu, and Lys⁶⁶₃-Glu had much lower activities (0.018-0.040 min⁻¹) than that of the wild type.

To test the role of important ionic amino acids in the catalytic function, we further purified Lys⁴⁴¹-Glu, Lys⁴⁶⁶-Glu, Lys⁴⁷⁰-Glu, Lys⁴⁸⁵-Glu, and Arg⁶⁴⁶ of P450cam (27). All mutants were purified as the high spin form (27). The triple mutant Arg¹³⁵-Leu + Arg¹³⁶-Leu + Arg¹³⁷-Leu could not be purified because the heme dissociated from the protein during the purification procedure. Activities of all purified mutants toward 7-ethoxycoumarin, which were obtained at 25°C to avoid denaturing some mutants (cf. "Experimental Procedures"), were much lower than that of the wild type (Table I).

Dissociation constants ($K_d$) of the reductase from the mutants were obtained by the Soret absorption spectral change. $K_d$ values (103-217 nM) of the mutants Lys⁴⁴¹-Glu, Lys⁴⁶⁶-Glu, Lys⁴⁷⁰-Glu, Lys⁴⁸⁵-Glu, and Arg⁶⁴⁶ were more than twice of that (47 nM) of the wild type, while those (<28-37 nM) of the mutants Lys⁴⁸⁵-Glu, Arg⁶⁴⁶-Glu, and Lys⁴⁸⁵-Glu were comparable to or less than that (47 nM) of the wild type.

To test whether electrons transfer to the heme of P450₄₄₀ through the reductase, we observed the Soret peak at 447 nm of the reduced P450₄₄₀-CO form in the presence of NADPH and triple amounts of the reductase. The peak at 447 nm of the wild type-reductase-CO solution quickly appeared after adding NADPH, while those of all mutants slowly appeared after more than 1 min. The rate of the appearance of the peak at 447 nm, which corresponds to the rate of the reduction of the heme, was estimated with a stopped flow spectrometer. Rate constants (<0.001-0.054 s⁻¹) of the reduction of the heme by NADPH in the presence of mutants of the reductase were much lower than that (5.9 s⁻¹) of the wild type.

DISCUSSION

Nelson and Strobel (22) suggested that there are several ionic domains on the surface of P450s. Stayton et al. (17) suggested from the molecular model of P450cam and cytochrome b₅ that basic amino acids such as Arg², Arg¹²⁷, Lys⁴⁴¹, and Arg⁶⁴⁶ of P450cam will be involved in the interaction with putidaredoxin. As observed in Fig. 1 (22), Arg² of P450cam corresponds to Lys⁴⁴¹ of P450₄₄₀, P450₄₄₀ has two other Lys residues, Lys⁴⁸⁵ and Lys⁴⁸⁵, in this region (Fig. 1). It seems that this region may be one of the ionic domains of membrane-bound P450₄₄₀ (22). Thus, we reversed the ionic character by changing these amino acids to Glu.

Arg¹³⁵ of P450cam corresponds to Arg¹³⁷ of P450 (Fig. 1) (22). This region of eukaryotic P450s has the Arg cluster, such as Arg¹²⁷-Arg¹³⁶-Arg¹³⁷ for P450s. Thus, we made three single mutants, Arg¹²⁷-Leu, Arg¹³⁶-Leu, and Arg¹³⁷-Leu, and one triple mutant, Arg¹²⁷-Leu + Arg¹³⁶-Leu + Arg¹³⁷-Leu. Since it seemed that the Arg¹³⁵-Glu type mutant may form self-ionic bond(s) on the protein surface of P450₄₄₀, we purposely changed Arg to a nonpolar amino acid Leu for the mutants of this region.

It was reported that Arg²⁷⁷ of P450cam (number for P450s) is essential for steroid 21 hydroxylase, since Trp and Leu mutants of this Arg have no activities (29). However, this Arg is conserved only for P450₁₇α and P450₂₁ (Fig. 1) (22). One notices that a highly conserved Arg is located at the upper part toward the amino terminus by two amino acid residues, which corresponds to Arg²⁷⁷ for P450α or to Arg²⁰⁰ for P450cam (Fig. 1) (22). We thus replaced this conserved Arg with Trp and Leu.

Fig. 1. Selected amino acid sequences of P450s (22, 23). Boldface letters of P450α are amino acids mutated in the present study. The center column of the lower part is a sequence alignment modified by us.
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Table I

| P450 type | Turnover number* | Microsomes | Purified | $K_d$ | Rate constant* |
|-----------|------------------|------------|----------|-------|----------------|
| Wild type | 0.674            | 0.380      | 47       | 5.9   |                |
| pAM82     | 0.005            |            |          |       |                |
| Lys<sup>440</sup>-Glu | 0.045 | 0.018 | 103 | 0.024 |                |
| Lys<sup>449</sup>-Glu | 0.019 | 0.012 | 208 | 0.010 |                |
| Arg<sup>434</sup>-Leu | 0.110 | 0.018 | <28 | 0.054 |                |
| Arg<sup>439</sup>-Leu | 0.620 |          |        |       |                |
| Arg<sup>438</sup>-Leu | 0.223 |          |        |       |                |
| Arg<sup>439</sup>-Leu | 0.302 |          |        |       |                |
| Arg<sup>434</sup>-Leu + Arg<sup>439</sup>-Leu | 0.042 |          |        |       |                |
| Arg<sup>434</sup>-Leu |- |          |        |       |                |
| Arg<sup>439</sup>-Leu |- |          |        |       |                |
| Lys<sup>467</sup>-Glu | 0.042 | 0.008 | 217 | 0.010 |                |
| Lys<sup>467</sup>-Glu | 0.013 | 0.032 | >200 | 0.033 |                |
| Arg<sup>467</sup>-Glu | 0.020 | 0.015 | <28 | <0.001 |                |
| Lys<sup>467</sup>-Glu | 0.040 | 0.015 | 37 | <0.001 |                |

* Turnover numbers were expressed by nmol min<sup>-1</sup> (nmol P450)<sup>-1</sup>. Turnover numbers of microsomal mutants were obtained at 37 °C (34), while those of purified mutants in the presence of P450 reductase were obtained at 25 °C in the presence of DLPC because some purified mutants such as Lys<sup>456</sup>-Glu and Lys<sup>453</sup>-Glu were not stable at 37 °C (cf. "Experimental Procedures"). Concentrations used for activities were 0.2 μM P-450<sub>450</sub>, 500 μM 7-ethoxycoumarin, 0.6 μM reductase, and 1 mM NADPH. By titrating the reductase, this concentration of the reductase was sufficient to have full activity of the wild type P450<sub>450</sub>, which is comparable to that previously reported (24, 31). For catalytic activities of purified mutants, 5 mg of DLPC/ml of buffer solution was sonicated until the opaque solution was changed to transparency. We always kept the DLPC solution for more than 2 h at 25 °C after we added the enzymes to the DLPC solution to ensure equilibrium (7). Buffer solutions consisted of 20 mM EDTA, 20 mM dithiothreitol, 4% glycerol, and 0.1 M potassium phosphate (pH 7.2). Experiments were repeated at least three times, and their averaged values are described. Experimental errors were less than 20% and less than 10% for the microsomal solutions and for the reconstituted solutions, respectively.

<sup>1</sup> Apparent dissociation constants ($K_d$) of the reductase were expressed by nM and were obtained from the Soret spectral change caused by adding the reductase to the P450<sub>450</sub>-acetanilide solution at 25 °C. The spin state of the high spin type P450<sub>450</sub> was converted partially to the low spin state by adding 20-30 mM acetanilide. The low spin portion of the P450<sub>450</sub>-acetanilide solution was changed back to the high spin state with two isosbestic points around 400 nm and around 425-460 nm by adding the reductase. Double reciprocal plots of the spectral change versus the concentration of the free reductase formed a straight line, indicating that a 1:1 reductase-P450<sub>450</sub> complex is formed. Thus, we used these Soret spectral changes accompanied with the spin change for obtaining $K_d$ values. We always kept the P450<sub>450</sub>-reductase solution for more than 40 min at 25 °C in each titrating step to reach equilibrium (3). Estimation of $K_d$ less than 28 nM was not feasible because the concentration of free reductase could not be obtained under these conditions. Buffer solutions containing sonicated DLPC were the same as those used for obtaining activities. For sample solutions a 1-cm cell was used, while two 5-mm cells placed in tandem were used for reference solutions to each cell P450<sub>450</sub>, and the reductase was added separately. Experiments were repeated at least three times and their averaged values are described. Experimental errors were less than 20% except for the mutant Lys<sup>467</sup>-Glu in which the correct value was not estimated due to very small spectral changes.

<sup>1</sup> Rate constants were expressed by s<sup>-1</sup> and were obtained by monitoring the Soret spectral peak at 447 nm of the CO-reduced forms of P450<sub>450</sub> at 25 °C. Rate constants were estimated from the fast phase. Since the observed kinetic tracing was the sum of the absorbance changes produced by reduction of both P450<sub>450</sub> and the reductase, the absorption contribution from P450<sub>450</sub> was determined by subtracting the spectrum produced by the reductase from that produced by the complete system. Reduction was initiated by adding 40 μl of the NADPH (1 mM)-DLPC (32 μM)-CO (approximately 0.8 mM) solution to 40 μl of the P450<sub>450</sub> (0.2 μM)-reductase (0.6 μM)-DLPC (32 μM)-CO (approximately 0.8 mM) solution. The stopped flow spectrometer with micro mixing cells has a dead time, 4.4 ms, a cell path, 10 mm and minimal volume for one shot, 30-40 μl. Both buffer solutions were the same as used for measuring activities. Opaque solution containing DLPC was sonicated until the solution became transparent, and then the enzymes were added to the solution. Before the reduction was started, 30 mM glucose, 50 units/ml glucose oxidase, and 1,000 units/ml catalase were added to both the P450<sub>450</sub> and NADPH solutions to eliminate oxygen gas and H<sub>2</sub>O<sub>2</sub> solved in the solutions. The DLPC solutions were always maintained more than 2 h at 25 °C to reach equilibrium after the enzymes were added (3). Experiments were repeated at least three times, and their averaged values are described. Experimental errors were less than 10%.

<sup>2</sup> Microsomes of yeast AH22 harboring a shuttle vector, pAM82, lacking P450<sub>450</sub> cDNA.

<sup>3</sup> Heme did not bind to the mutant protein.

<sup>4</sup> Ref. 24.

Lys<sup>401</sup> is highly conserved for all P450s (Fig. 1) (22). It was claimed that this Lys is involved in the interaction with the reductase (13, 15). Thus we made the Lys<sup>401</sup>-Glu mutant. Arg<sup>429</sup> of P450<sub>450</sub> nearly corresponds to Arg<sup>429</sup> of P450<sub>450</sub> (Fig. 1, left column of lower part). The heme did not bind to the mutant Arg<sup>429</sup>-Leu protein in our previous study (24). However, when one modifies amino acid alignment of this region as the center column of the lower part in Fig. 1, several basic amino acids such as Arg or Lys are modestly conserved in this region. Lys<sup>344</sup> of P450<sub>450</sub> perhaps corresponds to Lys<sup>460</sup> (Fig. 1). We changed Lys<sup>460</sup> and Arg<sup>455</sup> to Glu. A mutant Lys<sup>463</sup>-Glu was already made in our previous study (24).
Arg^{464} of P450_{can} corresponds to Lys^{465} of P450_d (Fig. 1) (22). The basic amino acid of this region is well conserved as Arg or Lys (Fig. 1) (22), leading to the Lys^{464}-Glu mutant.

We selected 7-ethoxyquinolinium as a substrate because catalytic activity of P450 toward this substrate is less influenced by change(s) of the ternary structure of the substrate binding site caused by mutations (24, 28). Thus a marked decrease in activity toward this substrate caused by mutations (Table I) will be associated with intrinsic and/or essential function for catalytic activity of P450_d. Thus, one may rule out the possibility that a structural change of the substrate binding site caused by the mutation is a reason for the decrease in activity of microsomal and purified mutants.

Since the heme did not bind to the Arg^{373} and Arg^{429} mutants, these amino acids may be so important to retain appropriate tertiary protein structure of P450_d to as hold the heme. Similarly the conserved Lys^{461} does not appear to be directly involved in the essential function of P450_d.

Affinities of the reductase to the Lys^{395}-Glu, Arg^{455}-Glu, and Lys^{463} mutants were comparable to or higher than that to the wild type (Table I), even if ionic characters of these amino acids were reversed. Thus decreased catalytic activities and rate constants of the heme reduction of these mutants were reversed. Thus decreased catalytic activity of P450_d toward this substrate caused by mutations (Table I) in addition to orient proper geometry of different proteins.

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In conclusion, Arg^{464} Lys^{465}, Lys^{465}, and Lys^{463} and Arg^{455} Lys^{463} often appear very important for the catalytic function of P450_d, probably by participating cooperatively in forming an electron transfer complex with the reductase. Ionic regions of the surface of the P450_d molecule which directly interact with the reductase may be similar, if not identical, to those of P450_{can} which directly interact with putidaredoxin.

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