Probing the Interactions of Putidaredoxin with Redox Partners in Camphor P450 5-Monoxygenase by Mutagenesis of Surface Residues*

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The role of surface amino acid residues in the interaction of putidaredoxin (Pdx) with its redox partners in the cytochrome P450*cam (CYP101) system was investigated by site-directed mutagenesis. The mutated Pdx genes were expressed in Escherichia coli, and the proteins were purified and studied in vitro. Activity of the complete reconstituted P450*cam system was measured, and kinetic parameters were determined. Partial assays were also conducted to determine the effect of the mutations on interactions with each redox partner. Some mutations altered interactions of Pdx with one redox partner but not the other. Other mutations affected interactions with both redox partners, suggesting some overlap in the binding sites on Pdx for putidaredoxin reductase and CYP101. Cysteine 73 of Pdx was identified as important in the interaction of Pdx with putidaredoxin reductase, whereas aspartate 38 serves a critical role in the subunit binding and electron transfer to CYP101.

Multiprotein redox enzyme systems such as methane monooxygenase, cytochrome P450s, and dioxygenases of similar molecular architecture are being investigated as biocatalysts for conversion of organic substrates with no functional groups into oxygen-bearing compounds with high regio- or stereo-selectivity. Maximizing the catalytic efficiency of such systems requires knowledge of the pathways of electron transfer and of the surface regions and amino acid residues involved in the interaction of the redox partner subunits.

Cytochrome P450*cam (CYP101) has been intensively investigated for over 20 years as a model P450 system (1). This soluble P450 (from Pseudomonas putida) consists of three subunits: putidaredoxin reductase (PdR, \(^{1} M_c \approx 43,500\)), putidaredoxin (Pdx, \(M_c \approx 11,600\)), and cytochrome P450*cam hydroxylase (CYP101, \(M_c \approx 45,000\)). The genes, camA (PdR), camB (Pdx), and camC (CYP101) from the cam operon have been cloned and sequenced, and the protein subunits were expressed in individual clones (2–5). Structural information is available for two of the three subunits of the CYP101 system. CYP101 has been crystallized in a number of states, and the structure is well defined (6–8). Structural information on Pdx comes from solution \(^{1}H\) NMR studies by Pochapsky and co-workers (9–11), and they have proposed a model.

Electron transfer in this system proceeds from NADH via the flavin group of PdR to the 2Fe-2S center of Pdx and then to the heme iron of CYP101 which accepts one electron at a time from Pdx. Because the details of the electron transfer pathway from one subunit to the next are missing, it is not known exactly how the subunits bind for the transfer of electrons. Ionic strength is well known to have an effect on binding and electron transfer suggesting that salt bridges are important in these interactions (12, 13). The role of some amino acid residues, specifically Trp-106 on Pdx and Arg-112 on CYP101, is known to be important for binding and electron transfer (14–18). Residues involved in the PdR-Pdx interaction are not necessarily the same as those in the Pdx-CYP101 complex. Site-directed mutagenesis of amino acid residues on adrenodoxin, a mitochondrial analog to the camphor hydroxylase, has implicated overlapping but not identical sites for binding of adrenodoxin to its redox partners in the P450sc system (19–21).

Recently, Sibbesen et al. (22) developed a functional biocatalyst from a multi-enzyme system by preparing fusion proteins of the CYP101 system. They linked the three genes in two different sequence orders (PdR-CYP101-Pd and PdR-Pdx-CYP101). Their results showed that the system was not optimal with respect to the rate of product formation. They noted that details of subunit interaction were still obscure. Information on surface interaction sites will lead to further optimization of such self-sufficient systems.

In this study we have used site-directed mutagenesis to alter surface residues of Pdx and tested the effect of these mutations in the reconstituted complete CYP101 system as well as on interactions of Pdx with specific redox partners. The purpose is to identify residues that are important for binding and electron transfer and to probe the degree of overlap of the binding sites of PdR-Pdx and Pdx-CYP101.

EXPERIMENTAL PROCEDURES

The three protein subunits of the P450*cam system were obtained by heterologous expression of the genes in separate Escherichia coli (DH5\(\alpha\)) clones. The clones were kindly provided by Dr. J. A. Peterson (University of Texas Southwest Medical Center, Dallas, TX). The gene for Pdx along with some 5' and 3'-flanking DNA was cloned between a HindIII and Smal site in the polycoding region of pBluescript (5). Mutants of the Pdx gene were obtained by a site-directed mutagenesis protocol (Transformer\(^{\text{TM}}\), CLONTECH, Palo Alto, CA).\(^{2}\) Mutations in the Pdx gene were identified by sequencing both strands of the gene. Sequencing reactions were performed using dye-linked deoxy terminator chemistry (Prism\(^{\text{TM}}\) Dye-Deoxy Terminator kit, Perkin-Elmer) in a thermo-

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\(^{1}\) The abbreviations used are: PdR, putidaredoxin reductase; camC, camphor P450 5-Monooxygenase by Mutagenesis of Surface Residues.

\(^{2}\) Certain commercial instruments, reagents, or materials are identified in this paper to specify adequately the experimental procedures. Such identification does not imply recommendation by the National Institute of Standards and Technology, nor does it imply that the materials are necessarily the best available for the purpose.
cycler (Perkin-Elmer model 9600). The products of the reaction were separated by electrophoresis and analyzed using a DNA sequencer (ABI model 373A DNA Sequencer, Perkin-Elmer). Individual clones, expressing PdR, CYP101, plus wild type and mutant Pdx, were grown in batch culture on undefined rich medium. Protein was released into the supernatant by lysis of the harvested cells, purified in two to three chromatographic steps, and the purity of every batch was monitored spectrophotometrically. The purity of some mutants were confirmed with two-dimensional gel electrophoresis. The details of the above procedures have been recently described (24). Purified protein subunits were stored at -80°C. Protein preparations were concentrated using centrifuugal membrane concentrators (Amicon Inc., Beverly, MA; Millipore Corp., Bedford, MA), and the buffer was exchanged using a Sephadex G-10 (Pharmacia Biotech Inc.) column prior to analysis. 

**Mass Spectral Analysis of the Mutant and Wild Type (WT) Pdx Proteins**—Mass spectra were recorded on a Finnigan MAT (San Jose, CA) TSQ-70 triple quadrupole mass spectrometer upgraded with a TSQ-700 operating system and equipped with an Analytica (Branford, CT) electrospray source. In the positive ion mode, mass spectra were obtained by scanning the first quadrupole from 1000 to 2000 m/z in 2 s at a sampling rate of five data points per mass unit. The charge state distribution observed in the electrospray mass spectra were deconvoluted using Finnigan's BIOMASS software. Samples were introduced into the electrospray source by capillary reversed-phase high performance liquid chromatography using a 0.52 × 1200-mm column packed with Vydac C8, 30-nm pore size (The Separations Group, Hesperia, CA). The capillary column was slurry-packed with the aid of a stainless steel pressure vessel (25) pressurized to 8300 kilopascal with helium. An Ultra-Plus binary gradient micro-LC pump (Microtech Scientific, Sunnyvale, CA) was used to produce the solvent gradients needed for the capillary reversed-phase high performance liquid chromatography. The pump was operated at 40 μl/min, but the column flow rate of 1–2 μl/min was obtained by pre-injector splitting. A Rheodyne (Cotati, CA) model 7520 injector with a 1-μl rotor was used. Prior to LCMS analysis, desalted Pdx samples were diluted to approximately 0.2 g/liter with water.

**Enzyme Assays**—The complete camphor monoxygenase reaction was conducted with all three subunits. The reaction contained Pdx (5 μmol/liter), PdR (0.5 μmol/liter), CYP101 (0.5 μmol/liter), NADH (330 μmol/liter), camphor (550 μmol/liter), KCl (100 mmol/liter) in potassium phosphate buffer (20 mmol/liter), pH 7.4. All components were prepared as stock solutions in the phosphate/KCl buffer. The reaction was started with the addition of NADH. The oxidation of NADH was monitored at 340 nm, and the oxidation of NADH was based on a molar absorptivity of 6.92 (mmol/liter)-1 cm-1. Kinetic studies were done with the complete system keeping the Pdx concentration constant at 5 μmol/liter. The concentration of the redox partner under investigation was varied, and the other partner was present at 0.5 μmol/liter. The kinetic data was analyzed using nonlinear regression analysis (Sigmaplot, Jandel Scientific, San Rafael, CA).

Two partial reaction assays, 1) the reduction of Pdx by PdR and 2) the oxidation of reduced Pdx by CYP101, were also performed. These were based on the absorbance difference between oxidized and reduced Pdx at 455 nm (molar absorptivity at 455 nm of reduced Pdx, ε = 2.6 mmol/liter-1 cm-1; and of oxidized Pdx, ε = 10.4 mmol/liter-1 cm-1). A large amount of Pdx was combined with a catalytic amount of PdR or CYP101. This allowed the reaction to proceed at a linear rate measurable over 0.2 min to 0.6 min. Because of the sensitivity of the reaction to small differences in the quantities of redox partners (PdR or CYP101), the results with mutant Pdx proteins were always compared with assays done with the WT Pdx performed on the same day and with the same preparations of other reaction components. The reaction mixture for Pdx reduction consisted of Pdx (100 μmol/liter), NADH (100 μmol/liter), and PdR (10 mmol/liter) in the phosphate/KCl buffer. The reaction was started with the addition of NADH. Absorbance changes were monitored at 455 nm.

In the assay monitoring Pdx oxidation, it was necessary to reduce Pdx prior to adding CYP101 and camphor. This was accomplished by using the same reaction mixture that was used for the Pdx reduction assay, raising the concentration of PdR to 20 mmol/liter. After an initial decrease in absorbance of Pdx was observed, resulting from the reduction of Pdx, a steady state absorbance reading was maintained for 1 to 2 min until NADH had been depleted. Then reoxidation of Pdx was observed by linear rate of increase in Pdx absorbance. The rate of nonenzymatic reoxidation remained linear over 0.5 to 1 min and was the same for mutants and WT Pdx, except for W106E (1.5 × WT), and for D38N (2 × WT). Once the pattern and rate of nonenzymatic reoxidation was established for WT and mutant Pdx, assays were started and observed to the point where NADH had been depleted. Then CYP101 (10 mmol/liter) and camphor (200 μmol/liter) were added, and the linear portion of the resulting Pdx reoxidation was measured. All values for total reoxidation were corrected for nonenzymatic reoxidation to determine the enzymatic rate of Pdx reoxidation.

**RESULTS**

A ribbon model of the structure of Pdx, as proposed by Pochapsky et al. (10) and based on NMR studies, is shown in Fig. 1. The side chains of the residues mutated in this study are also indicated. The 2Fe-2S cluster, at the middle portion of the molecule as depicted, has four cysteine ligands as follows: Cys-39, Cys-45, Cys-48, and Cys-86 (26). The first three lie on a loop leading from the helix on the right (amino acid residues 23–31). The fourth cysteine ligand (Cys-86) is present on another strand coming off a helix, which ends at Cys-73, on the left hand side of the molecule. This helix (residues 65–73) has several acidic residues that were shown in chemical modification studies to be involved in the binding of Pdx to the reductase (27). We chose to mutantize Cys-73 at the end of this helix. The loop containing three of the four cysteine ligands for the 2Fe-2S center has two acidic residues, Asp-34 and Asp-38. These would be candidates for electrostatic interaction with the series of basic residues in the vicinity of the heme group of CYP101. Recently, Pochapsky et al. (28) proposed that these two aspartates and the e-terminal carboxylate of Trp-106 interact with three arginines (Arg-72, Arg-109, and Arg-112) of CYP101. In our studies the acidic residues at positions 34 and 38 were replaced with the amide forms, which neutralized the charge, but did not change the overall size of the side chain. A second substitution of a nonpolar residue was done at position 38 (D38E), and cysteine was substituted for serine at position 42 which is also in the region near the Fe-S center of Pdx. Trp-106 has already been shown to have an important role in the Pdx-CYP101 complex with the most important aspect involving the aromatic nature of the amino acid (14, 15). The mutant W106E is similar to one (W106D) previously studied by Davies and co-workers (14, 15). In addition, three residues were chosen to test the hypothesis that they lie outside the interaction surface of Pdx with its redox partners and therefore could serve as anchors, the charge was neutralized on His-8 by replacement with a tyrosine; the charge was reversed on Asp-95 by replacement with a histidine; and the size of the side chain at Ala-55 was increased by replacement with a valine.

Mutant Pdx species were produced by a site-directed mutagenesis protocol. The identity of all of the mutations were verified two ways: 1) both strands of the mutated gene were

![Fig. 1. Ribbon representation of Pdx based on the NMR solution structure deposited by Pochapsky et al. (10) in the Protein Data Base.](image-url)
sequenced and 2) the molecular weight of each mutant Pdx protein species was determined by electrospray mass spectrometry (Table I). The mutated Pdx proteins were expressed in *E. coli* (DH5α) cells on the plasmid PIBI25 as was used for expression of the WT. The level of expression of the mutants in *E. coli* varied from the wild type. In most cases, the yield of protein (after purification) was somewhat lower than WT Pdx (3.6 mg of pure protein/g of cell FW). However, C73R expressed at higher levels than WT. The mutant proteins were purified by the same protocol as used for the WT, and purity was monitored spectroscopically according to Gunsalus and Wagner (29). Wavelength scans of the mutants were identical to the WT with the exceptions noted below. Based on the ratios of absorbances at 455 and 275 nm, the mole fraction purity of WT and mutants were used to determine the activity of the mutant Pdx. Activity of a mutant, as a percent of WT activity, is based on the average WT Pdx activity, although not to the same degree as its interaction with CYP101. The W106E mutant exhibited a low rate of activity with CYP101 as would be expected from other studies where substitution of tryptophan with anything other than another aromatic residue reduced activity to very low levels (14, 15). The substitution of asparagine for aspartic acid at residue 38 had a significant negative effect on the interaction with CYP101 (Tables III and IV). The primary effect of the substitutions at Asp-38, Ser-42, and Trp-106 was on the interaction of reduced Pdx with CYP101. The W106E mutant exhibited a low rate of activity with CYP101 as would be expected from other studies where substitution of tryptophan with anything other than another aromatic residue reduced activity to very low levels (14, 15). The substitution of asparagine for aspartic acid at residue 38 had a significant negative effect on the interaction with CYP101 (Tables III and IV). The primary effect of the mutations at Cys-73 was seen in the interaction of Pdx with PdR but not with CYP101 (Tables III and IV). The primary effect of the mutations at Asp-38, Ser-42, and Trp-106 was on the interaction of Pdx with the CYP101. The W106E mutant exhibited a low rate of activity with CYP101 as would be expected from other studies where substitution of tryptophan with anything other than another aromatic residue reduced activity to very low levels (14, 15). The substitution of asparagine for aspartic acid at residue 38 had a significant negative effect on the interaction with CYP101 reducing the activity to 22% WT. Substituting the non-polar residue isoleucine for Asp-38 was even more detrimental, nearly eliminating activity with CYP101. The S42C mutation clearly affected the interaction of Pdx with CYP101, whereas that with PdR was 100% WT. The results of the partial assay measurements with the D34N Pdx mutant presented an anomaly in that there appeared to be a small effect on the interaction of reduced D34N Pdx with CYP101, whereas the activity of this mutant in the total camphor reaction was 30% lower than WT. The effects of the mutations of residues Asp-38, Asp-34, and Trp-106 were not restricted just to interaction with the CYP101 subunit, which provides some evidence for overlap of binding sites on Pdx for PdR and CYP101. The substitution of asparagine at Asp-34 and Asp-38 appeared to result in a small enhancement of activity with respect to PdR. However, the positive effect disappeared with the substitution of the non-polar isoleucine for Asp-38, which resulted in the loss of activity. W106E had somewhat impaired interaction with PdR (67% WT activity), although not to the same degree as its interaction with CYP101.
A Michaelis-Menten kinetic analysis was conducted on some of the mutant Pdx species to investigate further the nature of the changes in the interaction between redox partners. These studies were done with the complete camphor assay, measuring NADH oxidation (Table V and Fig. 3). In kinetic studies the reacting substrates are maintained in nonlimiting quantities except for the substrate under investigation. But in this study, the protein subunits were being treated as substrates. It was not possible, for example, to provide unlimited quantities of CYP101 while investigating the kinetics of the reaction with regard to PdR and still be able to measure a linear rate of CYP101 while investigating the kinetics of the reaction with WT Pdx. Maximum activity (V_max) of the uncharged mutants, C73S and C73G, was similar to the WT, whereas that of the C73R charged substitution was reduced by nearly half. D34N and S42C had K_m values that were similar to the WT whereas the concentration of Pdx and the other redox partner was held constant. The data were analyzed using nonlinear regression analysis. The determination for each kinetic parameter was completed three or more times. The values are averages and the number in parentheses is 1 S.D.

**TABLE V**

**Determination of apparent kinetic parameters of redox partners for mutant and wild type putidaredoxin**

For these experiments the camphor-dependent NADH oxidation was measured with the complete P450cam monooxygenase system as described under “Experimental Procedures.” The concentration of the redox partner under investigation was varied, whereas the concentration of Pdx and the other redox partner was held constant. The data were analyzed using nonlinear regression analysis. The determination for each kinetic parameter was completed three or more times. The values are averages and the number in parentheses is 1 S.D.

| Mutant putidaredoxin | Wild type putidaredoxin | Pdx reduced | Mutant Pdx activity as a % of WT activity |
|----------------------|-------------------------|-------------|------------------------------------------|
| Identity             | n mol min⁻¹             | %           | n mol min⁻¹                               |
| C73S                 | 23.3 (2.1)              | 77          | 30.2 (0.7)                               |
| C73G                 | 17.4 (1.8)              | 60          | 29.2 (0.5)                               |
| C73R                 | 4.3 (0.2)               | 15          | 27.7 (2.1)                               |
| S42C                 | 31.2 (1.7)              | 100         | 31.1 (1.1)                               |
| D34N                 | 30.6 (1.1)              | 112         | 27.3 (1.4)                               |
| D38N                 | 33.0 (4.0)              | 121         | 26.4 (2.4)                               |
| D38I                 | 7.9 (0.3)               | 27          | 20.1 (2.0)                               |
| W106E                | 18.1 (1.1)              | 67          | 27.1 (2.8)                               |
| D95H                 | 29.7 (0.7)              | 100         | 29.8 (1.1)                               |
| H5Y                  | 32.4 (1.2)              | 104         | 31.2 (1.0)                               |
| A55V                 | 29.3 (2.2)              | 101         | 27.9 (2.4)                               |
| **Overall average**  | **28.5 (2.5)**          |             |                                          |

**DISCUSSION**

**Pdx and PdR Interactions Site—Cysteine 73** and other residues on the left-hand helix of Fig. 1 are important for the affinity of the Pdx-PdR subunits. Two of the mutations at Cys-73 (serine and glycine) resulted in similar affinity, whereas the substitution of arginine for cysteine at residue 73 was very disruptive, nearly eliminating activity. We conclude that Cys-73 is important to Pdx-PdR interaction because of the large effect of the change in the side chain with the Cys-73 mutations. Calculations of the Connolly surface (solvent-accessible surface) of the side chains of serine and cysteine gave values of 45.8 Å² for serine and 54.5 Å² for cysteine. The serine what affected) redox partner, as the reaction depends on the presence of the other redox partner for which the interaction is ineffective.

The K_m values for PdR with respect to the Cys-73 mutant Pdx species were approximately 4–18-fold greater than that of the WT Pdx. Maximum activity (V_max) of the uncharged mutants, C73S and C73G, was similar to the WT, whereas that of the C73R charged substitution was reduced by nearly half. D34N and S42C had V_max values that were similar to the WT whereas the concentration of Pdx and the other redox partner was held constant. The data were analyzed using nonlinear regression analysis. The determination for each kinetic parameter was completed three or more times. The values are averages and the number in parentheses is 1 S.D.

**TABLE V**

**Oxidation of reduced putidaredoxin by P450 and camphor**

See legend for Table II.
side chain has the same basic shape as cysteine but a 16% smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²). Cysteine is more polar than serine although the smaller surface area, and C73G has an even smaller side chain (14.1 Å²).

The CYP101 enzyme system fusion protein recently produced by Sibbesen et al. (22) contributes information relevant to understanding the redox partner interaction sites. The most active of the fusions linked the subunits in the following order: PdR-Pdx-CYP101, wherein W106 of Pdx is bonded, via a peptide linker, to the amino-terminal of the CYP101 subunit. The catalytic activity of the PdR-Pdx-CYP101 fusion was increased upon addition of extra CYP101 subunits, indicating that there

The crystal structure of CYP101 demonstrates the presence of a series of basic residues (Arg-79, Arg-109, and Arg-112) in a concave surface near the heme group (8). The Asp-34 and Asp-38 residues of Pdx lie near its 2Fe-2S center and would be likely candidates for salt bridge interactions between Pdx and the arginines of CYP101 and thus were chosen as targets for mutagenesis in this study. In a very recent paper, a model of optimized interaction of Pdx with CYP101, based on a molecular dynamics simulation, was presented (28). In this model, three salt bridges were proposed as follows: Arg-112 (CYP101) and Asp-38 (Pdx), Arg-109 (CYP101) and Asp-34 (Pdx), Arg-79 (CYP101) and the terminal carboxylate group of Trp-106 (Pdx). The results of our studies show that there was an effect upon the Pdx-CYP101 interaction when an amide form of the native carboxylate side chain is substituted at the 34 and 38 positions. But the results of the two substitutions are not equivalent to each other. The effect of the D38N substitution is dramatic in that not only is there a large increase in $K_m$, but the $V_{\text{max}}$ has been significantly decreased, whereas the effect of the amide substitution at Asp-34 was of less consequence to Pdx-CYP101 interactions (Table V). The Arg-112 residue of CYP101, the proposed salt bridge partner of Pdx Asp-38, has also been shown by mutagenesis to be important for electron transfer between Pdx and CYP101 (16–18). Arg-112 is also hydrogen-bonded to the heme propionate chain and contributes to the stability of CYP101 (17). Together these results support the idea that the Asp-38 and Arg-112 pair may have a very important role in binding of Pdx-CYP101 and electron transfer between subunits. Since the change at Asp-34 had much less effect than the change at Asp-38, the salt bridge relationship may not completely account for the role of Asp-38. The Asp-38–Arg-112 pair may be a route for electron transfer. Recent computer simulations support this pathway.

**Overlapping Interaction Sites on the Pdx Surface for PdR and CYP101**—In this study we have shown that separate but overlapping areas of the Pdx molecule are important for interactions of Pdx with either PdR or CYP101. The data suggest that separate residues play key roles for binding with each redox partner and that some of these have a lesser affect on the other redox partner. The aspartate residues (Asp-34 and Asp-38) that affect interactions with CYP101 were not important for interactions with PdR. In fact, the apparent affinity of Pdx for PdR increased slightly upon the neutralization of the charge by substitution with amides, more so in the case of Asp-38 (Table III). The substitution at Trp-106 also interfered with the interaction of Pdx with PdR (although not to the degree that was seen with CYP101), and S42C and C73S, Gly, or Arg affected the interaction with only one redox partner.

The CYP101 enzyme system fusion protein recently produced by Sibbesen et al. (22) contributes information relevant to understanding the redox partner interaction sites. The most active of the fusions linked the subunits in the following order: PdR-Pdx-CYP101, wherein W106 of Pdx is bonded, via a peptide linker, to the amino-terminal of the CYP101 subunit. The catalytic activity of the PdR-Pdx-CYP101 fusion was increased upon addition of extra CYP101 subunits, indicating that there

$^3$ A. Roitberg, unpublished data.
was more electron donation capacity at Pdx then was utilized by the CYP101 subunit of the fusion protein. The ability of the Pdx subunit of the fusion protein to pass electrons to exogenous CYP101 means that the interaction of the three subunits of the fusion protein is unlikely to be in a rigid complex. This is consistent with a loose arrangement between the subunits of the native protein, with Pdx acting as a electron shuttle between PdR and CYP101. This loose arrangement would be a requirement if there were overlapping binding sites for PdR and CYP101 on Pdx, as suggested by this study.

In conclusion, we have shown that adjacent and overlapping regions function in the interaction of Pdx with its redox partners. Cys-73 (and its associated helix) is important in PdR-Pdx binding, whereas an adjacent area, comprising residues on a surface loop subtended by the Fe-S center of Pdx, is critical to the interaction of Pdx with CYP101. The mutations at Asp-38 forms a salt bridge with Arg-112 (CYP101) (28). We hypothesize that the Asp-38–Arg-112 pair may be involved in electron transfer between subunits, in addition to the subunit binding role. We are currently investigating this possibility using molecular biology and molecular modeling approaches.

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REFERENCES

1. Mueller, E. J., Loida, P. J., and Sligar, S. G. (1995) in Cytochrome P450 Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., ed) pp. 83–124, Plenum Press, New York
2. Koga, H., Rauchfuss, B., and Gunsalus, I. C. (1985) Biochem. Biophys. Res. Commun. 130, 412–417
3. Unger, B. P., Gunsalus, I. C., and Sligar, S. G. (1986) J. Biol. Chem. 261, 1158–1163
4. Koga, H., Yamaguchi, E., Matsunaga, K., Aramaki, H., and Horiuchi, T. (1989) J. Biochem. (Tokyo) 106, 831–836
5. Peterson, J. A., Lawrence, M. C., and Amarneh, B. (1990) J. Biol. Chem. 265, 6066–6073
6. Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., and Kraut, J. (1985) J. Biol. Chem. 260, 16122–16130
7. Poulos, T. L., Finzel, B. C., and Howard, A. J. (1986) Biochemistry 25, 5314–5322
8. Poulos, T. L., Finzel, B. C., and Howard, A. J. (1987) J. Mol. Biol. 195, 687–700
9. Ye, X. M., Pochapsky, T. C., and Pochapsky, S. S. (1992) Biochemistry 31, 1961–1968
10. Pochapsky, T. C., Ye, X. M., Ratnaswamy, G., and Lyons, T. A. (1994) Biochemistry 33, 6432–6432
11. Pochapsky, T. C., Ratnaswamy, G., and Patera, A. (1994) Biochemistry 33, 6433–6441
12. Hintz, M. J., and Peterson, J. A. (1981) J. Biol. Chem. 256, 6721–6728
13. Roome, P. W., Jr., Philley, J. C., and Peterson, J. A. (1983) J. Biol. Chem. 258, 2593–2598
14. Davies, M. D., Qin, L., Beck, J. L., Suslick, K. S., Koga, H., Horiuchi, T., and Sligar, S. G. (1990) J. Am. Chem. Soc. 112, 7596–7598
15. Davies, M. D., and Sligar, S. G. (1992) Biochemistry 31, 11383–11389
16. Koga, H., Sagara, Y., Yaoi, T., Tsujimura, M., Nakamura, K., Sekimizu, K., Makino, R., Shimada, H., Ishimura, Y., Yura, K., Go, M., Ikeguchi, M., and Horiuchi, T. (1993) FEBS Lett. 331, 109–113
17. Nakamura, K., Horiuchi, T., Yasukochi, T., Sekimizu, K., Hara, T., and Sagara, Y. (1994) Biochim. Biophys. Acta 1207, 40–48
18. Unno, M., Shimada, H., Toba, Y., Makino, R., and Ishimura, Y. (1996) J. Biol. Chem. 271, 17869–17874
19. Coghlan, V. M., and Vickery, L. E. (1991) J. Biol. Chem. 266, 18606–18612
20. Wada, A., and Waterman, M. R. (1992) J. Biol. Chem. 267, 22377–22382
21. Beckert, V., Dettmer, R., and Bernhardt, R. (1994) J. Biol. Chem. 269, 2568–2573
22. Sibbesen, O., De Voss, J. J., and Ortiz de Montellano, P. R. (1996) J. Biol. Chem. 271, 22462–22469
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Grayson, D. A., Tewari, Y. B., Mayhew, M. P., Vilker, V. L., and Goldberg, R. N. (1996) Arch. Biochem. Biophys. 332, 239–247
25. Moseley, M. A., Deterding, L. J., Tomer, K. B., Jorgenson, J. W. (1991) Anal. Chem. 63, 1467–1473
26. Gerber, N. C., Horiuchi, T., Koga, H., and Sligar, S. G. (1990) Biochem. Biophys. Res. Commun. 169, 1016–1020
27. Geren, L., Tuls, J., O’Brien, P., Millett, F., and Peterson, J. A. (1986) J. Biol. Chem. 261, 15491–15495
28. Pochapsky, T. A., Lyons, T. A., Kazanis, S., Arakaki, T., and Ratnaswamy, G. (1996) Biochimie (Paris) 78, 725–733
29. Gunsalus, I. C., and Wagner, G. C. (1978) Methods Enzymol. 52, 166–188