Identification of the Binding Site on Cytochrome P450 2B4 for Cytochrome b₅ and Cytochrome P450 Reductase*

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A model of cytochrome P450 2B4, which was constructed by homology modeling with the four known crystal structures of the cytochromes P450 2B4 (Chang, T.-T., Stiffelman, O. B., Vakser, I. A., Loew, G. H., Bridges, A., and Waskell, L. (1997) Protein Eng. 10, 119–129), was used to select amino acids predicted, by computer docking studies and numerous previous biochemical and site-directed mutagenesis studies, to be involved in binding the heme domain of cytochrome b₅. Twenty-four amino acid residues located on both the distal and the proximal surface of the molecule were chosen for mutagenesis. These 24 mutant proteins were expressed in Escherichia coli, purified, and characterized with respect to their ability to bind cytochrome b₅ and support substrate oxidation. Seven mutants, R122A, R126A, R133A, F135A, M137A, K139A, and K433A, all on the proximal surface of cytochrome P450 2B4 near the heme ligand, were identified that exhibited decreased ability to bind cytochrome b₅. All of the mutants except K433A are located in either the C or C* helices or their termini. In addition, these seven mutants and two additional mutants on the proximal surface of cytochrome P450, R422A and R443A, were shown to exhibit decreased binding to cytochrome P450 reductase. These studies indicate that the binding sites for cytochrome b₅ and cytochrome P450 reductase are, as predicted, located on the proximal surface of cytochrome P450 2B4 and are partially overlapping but not identical.

The versatility of these oxidases and their potential for industrial purposes has generated a great deal of interest in understanding their structure, function, and redox reactions. The reaction catalyzed by P450 is shown in Reaction 1.

\[
\text{RH} + \frac{1}{2} \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ \quad \text{(Reaction 1)}
\]

where RH is the substrate and ROH is the oxidized product. The enzymatic cycle includes substrate binding, first electron transfer, oxygen binding, second electron transfer, substrate oxidation, and finally product dissociation. The redox partners for the microsomal P450s are cytochrome P450 reductase (P450 reductase) which contains both a FAD and FMN cofactor and cytochrome b₅ (cyt b₅). The crystal structure of P450 reductase has recently been published; and the two domains of the enzyme have been individually expressed and characterized (5, 6). In contrast, the crystal structure of cyt b₅ has been known for many years but has just recently been refined (7, 8). The first and second electrons are donated to P450 by P450 reductase. Because of its redox potential (\(\approx +25\) mV), cyt b₅ can only donate the second electron to P450 (9). In fact, it has been suggested that cyt b₅ may be able to transfer the second electron to selected P450s even faster than P450 reductase, thereby decreasing the amount of superoxide produced (10–12).

In an attempt to understand more thoroughly the in vitro and in vivo regulation and mechanism of reduction of P450 by its redox partners, the functional binding site on cytochrome P450 2B4 (CYP2B4) for cyt b₅ and P450 reductase has been identified. A model of the microsomal CYP2B4 has been constructed based on the previously determined crystal structures of four bacterial P450s (1), and residues on both the proximal and distal face of CYP2B4 have been mutated and characterized. Herein, we report that, as predicted, the site for binding cyt b₅ and P450 reductase is located on the proximal surface of CYP2B4 where the heme comes closest to the surface (3, 13, 14).

EXPERIMENTAL PROCEDURES

Materials—Yeast extract and tryptone for Escherichia coli growth media were obtained from Difco. Restriction endonucleases, T₄ DNA ligase, and Vent DNA polymerase were obtained from New England Biolabs (Beverly, MA). Ampicillin, chloramphenicol, NADPH, isopropyl thiolgalactopyranoside, d-aminolevulinic acid, lysozyme, polyethylene glycol 9 lauryl ether, phenylmethylsulfonyl fluoride, Tergitol NP-10, hemin, and Reactive Red-agarse were purchased from Sigma. Aprotinin, benzamidine, bestatin, leupeptin, and pepstatin were purchased from Calbiochem (San Diego, CA). DNase I and RNase A were obtained from Boehringer Mannheim (Germany). Methoxyflurane containing 0.01% (v/w) butylated hydroxytoluene was purchased from Abbott Laboratories (Chicago, IL). Benzphetamine hydrochloride was a gift of the Upjohn Co. (Kalamazoo, MI). Dilaurylphosphatidylcholine was purchased from Serdary Research Laboratories (Englewood Cliffs, NJ). Bio-Gel HTP hydroxyapatite was obtained from Bio-Rad. DE52 cellulose.
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lose was purchased from Whatman (Maidstone, UK). DEAE-Sepharose Fast Flow resin was obtained from Amersham Pharma Biotech (Uppsala, Sweden). CYP2B4 B0 cDNA was kindly supplied by Dr. R. M. Philpot (NIEHS, Research Triangle Park, NC) (15). pCWOri plasmid DNA was the gift of Professor M. Waterman (Vanderbilt University School of Medicine, Nashville, TN) (16).

Construction of the Plasmids Used to Express CYP2B4 and Its Mutants—Unless otherwise specified, all DNA manipulations were performed as described (17). All mutations were confirmed by double-stranded cycle sequencing using an Applied Biosystems 373 Stretch Sequencer and Ampli-stranded cycle sequencing using an Applied Biosystems 373 Stretch Sequencer and Ampli.
pLW-P450 in one reaction, and site-directed mutagenesis to produce the plasmid pLW-P450-EKSX was accomplished using a Muta-Gene Phagemid in vitro mutagenesis kit from Bio-Rad. Following mutagenesis to form the internal restriction sites, KpnI/SpeI-, SpeI/EcoRI-, EcoRI/XbaI-, and XbaI/HindIII-digested fragments of the CYP2B4 gene were excised from pLW-P450-EKSX and subcloned into the phagemid vectors pET-23a (Novagen, Madison, WI) or pTZ18U (Bio-Rad) for further mutagenesis. To generate the 24 alanine mutants of CYP2B4, 24 oligonucleotide primers were synthesized. Sequences of the oligonucleotides are shown in Table I. Site-directed mutagenesis was again accomplished using a Muta-Gene Phagemid in vitro mutagenesis kit from Bio-Rad. Following mutagenesis, correctly mutated P450 DNA fragments were excised from the mutagenesis vectors and subcloned into the expression vector pLW-P450-EKSX for expression in E. coli.

Expression of Wild-type and Mutant CYP2B4 in E. coli—The appropriate CYP2B4 (both wild-type and mutated) containing plasmid DNA was transformed into E. coli JM109 (DE3) cells containing pLysS (Novagen, Madison, WI) and grown overnight at 30 °C on Luria-Bertani (LB) agar plates containing 1 mM thiamine, 100 μg/ml ampicillin, and 74 μg/ml chloramphenicol. 3 ml of LB medium containing 1 mM thiamine, 100 μg/ml ampicillin, and 74 μg/ml chloramphenicol were inoculated with a single transformed colony and grown overnight at 30 °C, 200 rpm. 2 ml of this culture were then used to inoculate 100 ml of terrific broth (18) containing 1 mM thiamine, 0.5 mM d-aminolevulinic acid, 100 μg/ml ampicillin, and 74 μg/ml chloramphenicol in a 500-ml Erlenmeyer flask. The cultures were grown at room temperature (approximately 25 °C), 120 rpm, until they reached an A600 of at least 4, and then IPTG was added to a final concentration of 0.1 mM to induce expression. The cultures were then incubated for a further 72 h at room temperature (approximately 25 °C), with shaking at 120 rpm. Overexpression of wild-type CYP2B4 was attempted on a larger scale (200 ml of medium in a 1000-ml Erlenmeyer flask and 750 ml in a 2800-ml Fernbach flask), but the yield of holo-P450 obtained was reduced by 50–70%.

Purification of Wild-type CYP2B4 from E. coli—Unless otherwise specified, all operations were performed at 4 °C. Cells expressing P450, from 4000 ml of cell culture medium, were harvested by centrifugation at 4000 × g for 25 min, and the pellet was resuspended in 60 ml of buffer A (100 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.3 μM aprotinin, 130 μM bestatin, 1 μM leupeptin, 1 μM pepstatin, 0.1 mg/ml lysozyme, 0.1 mg/ml RNase, 0.1 mg/ml DNase). The solution was stirred for 4 °C and then frozen at −80 °C for 1 h. After thawing, the cycle of stirring, freezing, and thawing was repeated twice for a total of three times. The cells were lysed by sonication in an ice/salt bath using a Vibra Cell High Intensity Ultrasonic Processor (Sonics and Materials, Inc., Danbury, CT) for a total of 6 min at 40% power in 30-s pulses, with 5 min for cooling after each pulse. Unbroken cells were removed by centrifugation at 30,000 × g for 15 min, and the supernatant was ultra-centrifuged at 105,000 × g for 1 h to pellet the “membrane fraction.” The supernatant was discarded, and the pellet was resuspended in 40 ml of buffer B (10 mM potassium phosphate, pH 7.4, 20% glycerol, 1 mM EDTA, 0.6% polyethoxyethylene 9 lauryl ether) by stirring for 2 h at 4 °C. After the pellet had been completely resuspended, polyethoxyethylene 9 lauryl ether was added dropwise to the solution to a final ratio of 1:5, milligram of detergent:milligram of protein. The solution was stirred for 2 h at 4 °C and subsequently centrifuged at 105,000 × g for 1 h. The P450-containing supernatant was loaded directly onto a Reactive Red-agarose column (100 nmol of P450/ml of Reactive Red-agarose) pre-equilibrated with 10 volumes of buffer B. After loading the P450-containing supernatant, the column was washed with buffer C (10 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.6% polyethoxyethylene 9 lauryl ether) by stirring for 3 h at 4 °C. The P450-containing supernatant was decanted and loaded directly onto a Reactive Red-agarose column (100 nmol of P450/ml of Reactive Red-agarose) pre-equilibrated with 10 volumes of buffer B. After loading the P450-containing supernatant, the column was washed with buffer C (10 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.3% Tergitol NP-10) at the same rate until the A280 was below 0.05. Subsequently, the column was washed with 4 column volumes of buffer C containing 100 mM NaCl to elute weakly binding proteins. CYP2B4 was eluted with buffer C containing 500 mM NaCl.
NaCl. Fractions containing P450 were combined, dialyzed overnight against 100 volumes of buffer C, and loaded onto a Bio-Gel HTP hydroxylapatite column (30 nmol of P450/ml of hydroxylapatite), pre-equilibrated with 10 volumes of buffer D (5 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA). After loading the CYP2B4 containing fractions, the hydroxylapatite column was washed with buffer D until the A
\textsuperscript{275}
 of the eluant was below 0.005. P450 was eluted using buffer E (500 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA), and the fractions containing P450 were combined and dialyzed overnight against 100 volumes of buffer F (50 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA). After dialysis, the fractions were concentrated to 100–300 μM P450 using an Amicon centricon apparatus and stored at −280 °C. The amount of P450 in intact E. coli and solutions was determined from the CO minus the reduced difference spectrum (16), using a few grains of sodium dithionite as the electron donor and an extinction coefficient of 91 mM
\textsuperscript{2} cm
\textsuperscript{2}
 for the absorbance difference at 450 minus 490 nm (19).

Purification of Mutant CYP2B4 from E. coli—Mutant P450 proteins were purified as described for the wild-type P450, except for mutants R122A, R126A, Y190A, and F115A which had a specific content of less than 9 nmol/mg protein after the Reactive Red column. With these mutants, an additional purification step was required. Combined fractions from the Reactive Red columns were dialyzed overnight against 100 volumes of buffer C and loaded onto a DEAE-Sepharose Fast Flow column (100 nmol of P450/ml of DEAE Sepharose), pre-equilibrated with buffer C. The eluant containing the mutant P450s which did not bind to the DEAE-Sepharose column was then loaded onto a Bio-Gel HTP hydroxylapatite column, as described for the wild-type P450.

| Mutant | Sequence* |
|--------|-----------|
| F115A  | CCG CTC CCC GTT GGC cgc GAT CAC TCC GTA TCC |
| R122A  | GAA TCT CCG AAG GGC tgc CCA GCG CTC CCC GTT |
| R125A  | GCC CAG GAA GAA TCT tgc AAG aGC CCG CCA GCG CT |
| R126A  | GGT GGC CAG GAA GAA ggc CCG AAG GGC CCG CCA |
| S128A  | CCG CAT GGT GGC GAG Ggc GAA TCT CCG AAGGGC |
| R133A  | CCC CAT CCG GAA GTC Cgc CAT GGT GCG CAG GGT |
| F135A  | CCC CCT CCC CAT CAT Cgc CCG CTC CAT GGT GGC |
| M137A  | CCC CAG GGC GAA GTC Cgc CAT GGT GCG CAG CAG |
| R138A  | CTC CTC CAC GCT ggc CCC CAT GCC GAA GTG |
| R140A  | GGC CTC CTC CAC ggc CCC CAT GCC GAA |
| F171A  | ACT AGT GAT TGA GTG cgc CAG CAA GTG GTC |
| Y190A  | GAA CTC GGG GTG gTT agc GTG AAA GCG TTC TCC |
| P203A  | GAG GGA AAA GGA GTG ggc GAA AAA GGG TTT GTG |
| H226A  | GGT GTC GGC AGG AAA ggc CTC TAG GAA GCC GGG |
| R276A  | GTC GCT TGG GTC ggc GTC GTG TTT TTT CAT GGC |
| P283A  | GAG GTT GAC GTG GTG cgc TTT TAG GAA GCC GGG |
| H335A  | GAG GGC CGG AGG GGC ggc GGA GAT CAT CTC |
| K421A  | AAA GCC TTC ATT CCT ggc CAG TGC CCC GTT GGC |
| R422A  | CAT AAA GCC TTC ATT ggc TTT TAG GAA GCC GGG |
| R422A  | CAT AAA GCC TTC ATT ggc TTT TAG GAA GCC GGG |
| K433A  | GCC CAG AGA AAT GCC ggc CCG CAG GAA GGA |
| R434A  | TCC GCC CAG ACA AAT Ggc CTT CCC CAG GAA GAA |
| R443A  | GAG GAA CAC CTC GGT ggc CTC TAG GAA GCC TTC GGC |
| P472A  | CAC GCC ACT CTC CCG ggc AGT GAG GTC GAT GTC |
| Y484A  | GAG GAA GGC GAT CTC ggc CTT CCC CAG GGC CAG GGA |

*The bases that were altered to introduce the indicated mutation are shown in lowercase letters.

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Purification of CYP2B4, Cyt b<sub>5</sub> and P450 Reductase from Rabbit Liver—Liver microsomes were prepared from phenobarbital-treated White New Zealand White rabbits (20). CYP2B4 was purified from rabbit liver microsomes as described previously (21) and had a specific content of 18 nmol of P450/mg of protein. NADPH P450 reductase preparations were purified from rabbit liver microsomes according to the method outlined (22) from rabbit liver microsomes. The effective concentration of the P450 reductase was calculated from its activity in the cytochrome c assay (10, 23). It typically had a specific content of 48 nmol of P450 reductase/mg of protein. Cyt b<sub>5</sub> was purified from detergent-solubilized rabbit liver microsomes as described previously (24) and had a specific content of 45–52 nmol of cyt b<sub>5</sub>/mg of protein. The concentration of cyt b<sub>5</sub> in crude preparations was determined by measuring the difference spectrum (reduced minus oxidized) using an extinction coefficient of 190 nm<sup>1</sup> cm<sup>-1</sup> (424 minus 409 nm) (25). In purified preparations an extinction coefficient of 117 nm<sup>1</sup> cm<sup>-1</sup> at 413 nm was used (26). Apocytochrome b<sub>5</sub> was generated by acetonitrile precipitation in the presence of HCl (27). Soluble cyt b<sub>5</sub> was produced by treating cyt b<sub>5</sub> with trypsin as described previously (28).

**Protein Analysis**—Protein concentration was determined using the BCA assay (Pierce) or by the Lowry method (29) after precipitation of the proteins in the presence of trichloroacetic acid and deoxycholate (30). Bovine serum albumin was used as a standard. SDS-polyacrylamide gels (9% for P450) and 15% for cyt b<sub>5</sub>, were used as described (31). Immobilon P transfer membranes (Millipore Corp., Bedford, MA) and polyclonal goat anti-rabbit CYP2B4 antibody at a 1:1000 dilution as the primary antibody (Oxford Biomedical Research, Oxford, MI) and horse-radish peroxidase-linked sheep anti-goat antibody (Sigma) at a 1:1000 dilution as the secondary antibody.

**Determination of the Equilibrium Dissociation Constant (K<sub>d</sub>) of the Cyt b<sub>5</sub>-CYP2B4 Complex**—The binding of cyt b<sub>5</sub> to CYP2B4 was determined by measuring the type I spectral change (decrease in absorbance at 420 nm plus the increase in absorbance at 385 nm) occurring when cyt b<sub>5</sub> is added to a solution of P450, methoxyflurane, and DLPC. Suspensions of DLPC in water (2–5 mg/ml) were sonicated using a microtip probe on a Vibra Cell High Intensity Ultrasonic processor (Sonics and Materials, Inc., Danbury, CT) for a total of 2 min at 40% power in 30–50 pulses, with 5 min cooling after each pulse, and then centrifuged at 13,000 × g for 5 min. P450 (0.3 nmol) in 50 μl of 100 mM potassium phosphate, pH 7.4, 20% glycerol, was mixed with DLPC (48 nmol) and preincubated at room temperature for 1 h. The solution was then adjusted to 1.0 ml using a solution of 100 mM potassium phosphate, pH 7.4, 20% glycerol which was saturated with methoxyflurane. The P450 solution was added to one chamber of a final displacement of 200 μl from a 1:1000 solution of CYP2B4 and incubated for 10 min at 37 °C. The other chamber contained 1.0 ml of a solution of cyt b<sub>5</sub> (0.14 to 4.0 μM) in 100 mM potassium phosphate, pH 7.4, and 20% glycerol which was saturated with methoxyflurane. The solutions were equilibrated to 25 °C, and the absorbance at 420 and 385 nm was recorded. The solutions were then mixed by inverting the cuvette. After mixing, the absorbance at 420 and 385 nm was followed until it stabilized (typically 10–15 min). The absorbance resulting from calcium chloride was subtracted from the final absorbance obtained in the absence of calcium chloride. Absorbance changes were determined using a spectrophotometric assay (33).

**Determination of the of the Cyt b<sub>5</sub>-CYP2B4 Complex**—The equilibrium dissociation constant for the benzphetamine–P450 complex (the spectral binding constant K<sub>b</sub>) and its mutants were determined using a spectrophotometric assay (35). P450 (0.72 nmol) was mixed with DLPC (176 nmol) in 100 mM potassium phosphate buffer, pH 7.4, 20% glycerol and incubated for 1 h at room temperature. The solution was diluted to 360 μl with 100 mM potassium phosphate buffer, pH 7.4, 20% glycerol which was saturated with methoxyflurane and equilibrated to 30 °C. The spectrum of the solution was recorded between 350 and 500 nm. Aliquots of a 10 mM benzphetamine hydrochloride solution were added, and the spectrum was recorded after each additional concentration of benzphetamine. Spectral changes were complete within 1–2 min of addition of benzphetamine. Final concentrations of benzphetamine were 10, 30, 100, 300, and 1000 μM. Spectra were corrected for dilution and differences in absorption of cyt b<sub>5</sub> between the experiments and a blank. A control experiment with benzphetamine at a concentration of 10 μM and equilibrated to 30 °C. A plot of benzphetamine concentration versus ΔA<sub>300nm</sub> gave a straight line, which was used to calculate the K<sub>b</sub> (slope/intercept with y axis).

**Determination of Methoxyflurane Metabolism**—The products of methoxyflurane metabolism (methoxydifluoroacetic acid and dichloroacetic acid) were measured by a gas chromatography-selected ion mass spectrometry assay, as described previously (10). The final concentration of P450, P450 reductase, and cyt b<sub>5</sub> in the reaction mixture of the wild-type and all mutant proteins was 0.2 μM. Experiments were performed twice in duplicate with 0.5 ml of reaction mixture for 5 min. Aliquots (200 μl) were removed after 1 and 6 min and were quenched with 140 μl of 30% H<sub>2</sub>S<sub>4</sub>O<sub>6</sub>. The amount of methoxyflurane metabolism occurring between 1 and 6 min was recorded. The internal standards containing H<sub>3</sub>-methoxydifluoroacetic acid and 2,2-[2-13C]dichloroacetic acid were added, and samples were stored at −80 °C before extraction and analysis by mass spectrometry. In addition, assays with the mutant proteins R122A, R126A, R133A, F135A, M137A, K139A, R422A, K433A, and R443A were performed twice in duplicate in a final volume of 2 ml (800 μl aliquots were removed after 1 and 11 min with 560 μl 30% H<sub>2</sub>S<sub>4</sub>O<sub>6</sub>). In order to find a constant intensity to allow by gas chromatography selected ion mass spectrometry.

**Identification of the Interprotein Surface Docking Regions of CYP2B4 and Cyt b<sub>5</sub>**—The method used to find surface regions for binding is a surface complementarity algorithm for protein docking (36, 37). The method, embodied in the program GRAMM, performs an exhaustive search for protein-protein surface complementarity. The
three original contact regions obtained using this method at low resolution (6.4–6.8 Å) have been described (1). In the studies reported here, this same method has been used for the CYP2B4-cyt b₅ pair but at the higher resolution of 3.4 Å. The results are more accurate than those from the previous 6.5-Å simulation but still can tolerate local inaccuracies in atomic details. The 10 lowest energy docking positions were analyzed and the results used to select preferred binding regions on CYP2B4 for cyt b₅.

RESULTS AND DISCUSSION

Optimization of the Expression of CYP2B4 and Its Mutants in *E. coli*—Previous overexpression of mammalian P450s has been achieved, using either derivatives of pCWori + (16) or the Amersham Pharmacia Biotech vector pKK223-3 (38), both of which use a tac promoter. CYP2B4 cDNA was initially cloned into both of these vectors, but when expression was attempted, CYP2B4 protein could not be identified in either Western blots of cell extracts or spectrophotometrically. A T₇ promoter containing vector, pET-23d, was then selected for further expression attempts. Initially, P450 was only observed on Western blots. However, reduction of the temperature to 28 °C after induction with IPTG resulted in the production of small amounts of holo-CYP2B4. To optimize the expression, different host strains, concentrations of δ-aminolevulinic acid, incubation temperatures, and incubation times were systematically tested. However, the maximum yield of holo-CYP2B4 obtained using this vector could not be increased above 200 nmol/liter.

To increase the yield, the low copy pBR322-derived origin of replication in pET-23d was replaced with that from the high copy pBluescript II KS + vector (ColEl origin of replication), to generate the expression vector pLW01 (Fig. 1). The level of holo-CYP2B4 expressed using this vector was increased at least 2-fold over that obtained from pET-23d and reproducibly gave 400–600 nmol of holo-P450/liter. To achieve optimal expression, it was necessary to grow the *E. coli* (both before and after induction with IPTG) at room temperature. The addition of 0.5 mM δ-aminolevulinic acid to the growth medium and extending the length of the incubation after induction to 72 h both resulted in an increase in CYP2B4 expression. Continuing expression beyond 72 h after induction did not result in a further increase in holo-P450 expression.

In order to overexpress significant levels of a number of the P450s in *E. coli*, it has frequently been necessary to mutate the N terminus of the protein as first described by Waterman and co-workers (16, 39, 40). In an attempt to optimize the expression of CYP2B4, two mutants were constructed, one in which the Ala codon GGT (which replaced a glutamic acid residue) in the second position E2A, and in addition F3L, S4L, R434A (which describe the rationale for the construction of the mutants. The numbers in this column correspond to the numbers in the text under “Rationale for the Mutation of Specific CYP2B4 Amino Acids” which describe the rationale for the construction of the mutants. The location of the mutated amino acids in the secondary structure of the cytochrome P450 2B4 model is given in parentheses in the first column. The secondary structure nomenclature is defined in Refs. 1 and 3. m-β indicates the meander to β-bulge. β indicates the β bulge. The capital letters indicate the helix in which the residue is located. Group A mutants bind cyt b₅ normally; group B mutants bind cyt b₅ poorly; groups C, D, and E mutants could not be characterized because the proteins were unstable, only apo protein was produced or protein was not produced, respectively.

| Group | Residue and location in secondary structure | Why amino acid mutated | Expression Specific content
|-------|---------------------------------|----------------------|-----------------------|
| Group A | mg P450/liter | nmol P450/mg protein |
| Wild type (E. coli) | 0 | 0 |
| 1 | 0 | 0 |
| R122A (B'-C') | 5 | 20–25 |
| F135A (C*) | 5 | 20–25 |
| M137A (C*) | 1, 5 | 20–25 |
| K139A (C*-D) | 5 | 20–25 |
| K433A (C) | 6 | 20–15 |
| R422A (m-β) | 6 | 20–25 |
| R443A (L*) | 0 | 0 |
| Y484A (I3-2) | 1 | 20–25 |

Group B

| Group C | Why amino acid mutated | Expression Specific content |
|--------|----------------------|-----------------------|
| P472A (β4-1) | | 1 |
| R125A (C) | 1, 2, 5 | 5 |
| F171A (E*) | 1 | 8 |

Group D

| Group D | Why amino acid mutated | Expression Specific content |
|--------|----------------------|-----------------------|
| F283A (turn before I) | | 1 |
| R494A (β) | 6 | 0 |

Group E

| Group E | Why amino acid mutated | Expression Specific content |
|--------|----------------------|-----------------------|
| S128A (C) | 4, 5 | no P450 |
| R140A (C*+D) | 1, 5 | no P450 |

The level of expression of the wild-type and mutant P450s is summarized in Table II.
coli was used as the starting point of the purification. A number of different detergents were tested for their ability to solubilize the membrane pellet. The best results were obtained using polyethoxylated ethylene 9 lauryl ether, which extracted approximately 90% of the holo-CYP2B4. The first purification step after solubilization of P450 from the E. coli membrane fraction has conventionally involved DEAE-ion exchange chromatography (42–47). However, with CYP2B4 only a small increase in specific content (e.g. from 1.0 to 2–3 nmol of P450/mg of protein) was achieved. The solubilized CYP2B4 was, therefore, purified using Reactive Red 120-agarose affinity chromatography. This single step resulted in extensive purification, with the specific content typically increasing from 1.0 to 11.4 nmol of holo-P450/mg of protein. Since cyt P420 did not bind to the resin, this column also separated cyt P420 from P450. The final step in the procedure was a hydroxylapatite column to remove detergent, which also resulted in a slight purification, yielding a specific content of 15.4 nmol of wild-type P450/mg of protein. Table III illustrates the overall recovery (15%) and purification achieved at each step in the P450 isolation procedure. The spectrum of wild-type CYP2B4 purified from E. coli is identical to that purified from rabbit liver microsomes (20).

The purification of the majority of the mutant P450 proteins was performed as described for wild-type CYP2B4. However, the F115A, R122A, and R126A mutant P450s had specific contents of below 6.5 nmol of P450/mg of protein after the Reactive Red affinity chromatography, so an additional purification step using DEAE chromatography was performed. This second column produced protein with a specific content of 7.9–12.1 nmol of P450/mg of protein. The overall yield from the cell culture (4×10–15%) was not significantly affected by this additional DEAE column. Sufficient quantities of the P450 mutant proteins, F171A and R125A, could not be isolated due to their instability during the purification procedure (90% was lost during solubilization of the membrane fraction, Table II).

**Rationale for the Mutation of Specific CYP2B4 Amino Acids**—Table II lists the residues in CYP2B4 selected for mutation, the rationale for their mutation, and their location in the secondary structure of the model of CYP2B4. In an effort to simplify the presentation of the data we may appear to discuss the model as though it were a crystal structure, although clearly the model is not a crystal structure. It is simply a detailed hypothesis that has been used to design and testively interpret the experiments described in this article. All residues were mutated to the most common amino acid, alanine, in order to evaluate the function of the amino acid side chain distal to the β-carbon. The selection of these residues was guided by computer docking studies of the heme domain of cyt b₅ and a model of CYP2B4 (1), and the considerable amount of experimental evidence which indicated that the redox partners of P450 would bind on its proximal face where the heme is closest to the surface (3, 13, 14). Column 2 of Table II also tabulates the reason a specific mutant was constructed. The numbers in column 2 of Table II correspond to the seven reasons provided in the following paragraphs. The group A mutants in Table II correspond to those that bind cyt b₅ normally, and the group B mutants are those that bind cyt b₅ poorly. Group C mutants produced holo-P450 in quantities inadequate for protein characterization. Groups D and E mutants produced apoP450 or no P450, respectively. The two group A mutants R422A and R443A that bind cyt b₅ normally but bind P450 reductase poorly are indicated by a superscript b. Thus Table II summarizes and collates why the mutants were constructed, which mutants were expressed, and which mutants bound cyt b₅ and P450 reductase poorly. The detailed characterization of the mutant proteins is provided in later sections of this report.

1. The five proximal region mutants R125A, R132A, M137A, R140A, and R443A of CYP2B4, the five distal region mutants F171A, Y190A, H226A, P472A, and Y484A, and the two side surface mutants F115A and P283A were selected partly based on predictions from the computer simulations at low and high resolution of complexes of CYP2B4 with cyt b₅. The x-ray structure of cyt b₅, and a model of CYP2B4 were used as input to the program GRAMM (37) that uses a surface complementarity algorithm to identify surface contact regions in the two protein partners of a protein-protein complex. The results of a high resolution study at 3.4 Å indicated a strong preference for a proximal binding region and eliminated the other two candidate regions on the distal and side surface of CYP2B4 found at low resolution (6.4–8.6 Å). Specifically, of the 10 lowest energy docked complexes obtained from GRAMM at 3.4 Å, six of them formed a tight cluster at the proximal binding region of CYP2B4. Such a cluster is a strong indication of the real binding site (37). The remaining four were spread randomly in different surface regions without forming clusters and were discounted as false positives. The residues of CYP2B4 found to be in close contact with residues of cyt b₅ in the three lowest energy representative configurations of these six proximal region complexes are shown in Fig. 4, A–C, and Fig. 5 illustrates the surface location of the amino acids chosen for mutation.

2. Arg-125, Lys-421, and Arg-443 on the proximal face were selected because they are homologous to CYP2B4 residues 121–134 (residues could inhibit the binding of cyt b₅ in the three lowest energy representative configurations of these six proximal region complexes are shown in Fig. 4, A–C, and Fig. 5 illustrates the surface location of the amino acids chosen for mutation. 125, Lys-421, and Arg-443 which had been predicted to form salt bridges with cyt b₅ and putidaredoxin (14). Arg-112 has also been shown to be important in electron transfer (48).

3. Arg-126 was selected for mutation because it is homologous to Arg-129 in cytochrome P450 2A5 (CYP2A5). In CYP2A5 (mouse P450c8) mutation of R129 to serine decreased the ability of CYP2A5 to bind to cyt b₅-conjugated Sepharose 4B (49).

4. Ser-128 was chosen for mutation because cyt b₅ has been demonstrated to be a competitive inhibitor of the phosphorylation of Ser-128 by cAMP-dependent protein kinase (50).

5. Arg-122, Arg-125, Arg-126, Ser-128, Arg-132, Phe-135, Met-137, Lys-139, and Arg-140 were chosen for mutation because of two previous studies. In one study, it was suggested that cytochrome P450 2B1 (CYP2B1) residues 116–134 (residues 116–134 in CYP2B4) were involved in binding cyt b₅ because a synthetic peptide composed of these CYP2B1 residues could inhibit the binding of cyt b₅ to CYP2B1 (51, 52). In the second study, Davydov and co-workers (53) predicted that CYP2B4 residues 121–145 would be involved in binding cyt b₅. This prediction was based on their sequence alignment studies that indicated that residues 68–87 of cyt c which are involved in cyt b₅ binding were homologous to CYP2B4 residues 121–145. Residues 125–139 constitute the C and C₇ helices (see

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**Table III**

| Purification step            | Volume | Halo P450 | Specific content | Yield |
|------------------------------|--------|-----------|-----------------|-------|
| 1. E. coli cell culture      | 1000   | 23.3      | 100             | 100   |
| 2. After sonication and removal of unbroken cells | 77     | 11.8      | 11.8            | 51    |
| 3. After solubilization of membrane fraction | 50     | 11.9      | 1.0             | 51    |
| 4. Reactive Red agrose 120 | 19     | 5.4       | 11.4            | 23    |
| 5. Hydroxylapatite         | 0.5    | 3.4       | 15.4            | 15    |
Refs. 1 and 3 for secondary structure nomenclature) which are on the proximal surface of CYP2B4 near the heme.

6. Arg-422, Lys-433, and Arg-434 were selected because they were basic residues near the heme that previous investigators had suggested might be involved in binding P450 reductase (54–56).

7. The basic residues, Lys-276 and His-335, were controls since they are not predicted to be in any surface contact region or identified in biochemical studies.

**Location in the CYP2B4 Model of Groups C, D, and E Mutants—** Four of the alanine mutants (S128A, R140A, F283A, and R434A) did not express holo-P450 protein (Group D and E in Tables I and IV). The location of the mutated residues and the hydrogen bonding pattern of their side chains in the model was studied in an attempt to gain insight into why the mutant proteins may not have expressed holoprotein (Table IV). Phe-283 is located on the distal face of the model, in the loop before the I helix with no obvious important structural role unless it stabilizes loop formation. Ser-128, Arg-140, and R434A are all located on the proximal face of the model. R140A, which does not express either holo- or apoprotein, is located on a loop between the C* and D helices with its side chain completely exposed on the molecular surface to water with no discernable structural role.

The side chain hydroxyl of Ser-128 on the C helix is buried and forms a hydrogen bond to the backbone carbonyl oxygen of Leu-124, the residue immediately preceding the beginning of the C helix. This hydrogen bond is possibly important for the...
structural stability of the protein. However, mutation of the homologous residue Ser-129 to alanine or glycine in murine P450 2E1 and expression of the mutant proteins in COS cells resulted in holoprotein (57). A buried Ser-128 side chain is consistent with the most recent studies of Schenkman and co-workers (50) that indicate that Ser-128 in CYP2B1 can only be phosphorylated in the apoprotein. The three mutants P472A, R125A, and F171A in group C in Tables II and IV could be expressed but could not be purified in sufficient quantity to characterize. The location of these mutated amino acids in the CYP2B4 model and the possible result of mutating the amino acid on the structure of CYP2B4 was examined in an attempt to understand why these mutants might be unstable (see Table IV). One of the side chain NH groups of Arg-125 (aligns with P450 camphor Arg-112) forms a hydrogen bond with the backbone carbonyl of Lys-433 and a buried water which, in turn, forms a hydrogen bond to the surface to solvent. The alanine mutant would be unable to form the hydrogen bonds with Lys-433 and the heme and the shorter alanine side chain would likely produce a cavity on the surface of the protein by which water could gain access to the heme and thereby decrease the heme-protein binding. A similar phenomenon was observed when Tyr-74 was mutated to lysine in cyt b₅ (59). At present, the poor expression and instability of P472A and F171A cannot be explained (Table II).

It is of interest that the remaining mutants gave rise to wild-type levels of protein expression and stability even though the percent solvent accessibility of the amino acid and its side chain is provided. These values were determined using VADAR: A Comprehensive Program Suite for Protein Structural Analyses by D.S. Wishart, L. Willard, F.M. Richards and B.D. Sykes, University of Alberta, Edmonton, Alberta, Canada.
Characterization of the Ability of Wild-type and Mutant P450s to Bind Cyt b₅—Table V lists the Kᵦ values of the wild-type and mutant P450s with cyt b₅. The wild-type protein has a Kᵦ of 0.02 μM in good agreement with a previously determined value of the Kᵦ of the cyt b₅-CYP2B4 complex (35). Mutants Y190A, K276A, H335A, K421A, R422A, R443A, and Y484A had a Kᵦ of 0.02 μM similar to that of the wild-type protein and served as negative controls. The Kᵦ of His-226 was 0.45 μM and was considered to be indistinguishable from wild type. The Kᵦ could not be determined for the F115A mutant protein because it precipitated in the cuvette. CYP2B4 residues K421A and R443A are homologous to cyt P450 camphor residues Lys-344 and Arg-364 which had previously been hypothesized to be involved in cyt b₅ and putidaredoxin binding (14, 60). The mutants R122A, R126A, R133A, M137A, K139A, and K433A were observed in initial experiments to have a higher Kᵦ than wild-type proteins (Table V). Repeat experiments using higher P450 (0.2–0.5 μM) and cyt b₅ concentrations (0.5–10 μM) were conducted in an attempt to obtain more accurate estimates of the Kᵦ values by using protein concentrations that would elicit at least 50% of the predicted ΔGₘₜ−wₑ₅. The data obtained with R122A and R133A covered the middle to upper part of the binding curve; the spectral changes with F135A and M137A plotted to the middle of the binding curve, whereas the absorbance changes observed with R133A and R126A only covered the lower part of the binding curve even with a P450 concentration of 0.5 μM. Therefore, the Kᵦ values of R133A and R126A are the most tentative. The ΔAₘₜ−wₑ₅ for all mutants was estimated from curve fitting and normalized to 0.15 μM P450. Within experimental error (data not shown) the ΔAₘ₅ₐₓ for all mutants was similar.

The seven amino acids that partially define the binding site on CYP2B4 for cyt b₅ are all located on the proximal surface near the heme ligand cysteine (Fig. 6A). All of the mutants except K433A are either located in the C or C* helices or in loops at the termini of these helices. These results are in striking agreement with the predictions by Davydov and co-workers (33) that CYP2B4 residues 121–145 would be involved in binding cyt b₅. The results also confirm the predictions of the surface complementarity algorithm which was able to localize the binding site to the proximal surface of CYP2B4 near the heme (37). However, the results do not substantiate the prediction that Lys-421 and Arg-443 would be involved in cyt b₅ binding because of their homology to residues Lys-344 and Arg-364 in P450 camphor which were hypothesized to participate in cyt b₅ binding (14).

Characterization of the Ability of Wild-type and Mutant P450s to Metabolize the Substrates Methoxyflurane and Benzphetamine—The results obtained with the cyt b₅ binding assay indicate that seven of the mutant proteins have decreased ability to bind cyt b₅. If the active site and substrate access channel have the same conformation as the wild-type protein, the binding and metabolism of substrates which do not require cyt b₅ for their metabolism should be similar to that observed for wild-type P450. In order to determine whether the CYP2B4 mutant proteins functioned like wild type, three assays were performed.

### Table V

| P450 2B4 residues and location in 2G structure | Kᵦ (P450-b5 complex) | ΔΔG mut-wₑ₅ | MF metabolism | Kᵦ (P450-BP complex) | Kᵦ (mut-BP complex) | ΔΔG mut-wₑ₅ | Predicted Vₘₕₐₓ BP metabolism |
|-----------------------------------------------|----------------------|---------------|---------------|-----------------------|---------------------|---------------|-----------------------------|
| P450 2B4                                 | μM ± S.D. | kcal/mol | μM ± S.D. | μM ± S.D. | kcal/mol |

*a The location of each residue in the secondary structure of cytochrome P450 2B4 is given in parentheses. The secondary structure nomenclature is provided in the footnote to Table II and Refs. 1 and 5.

*b ΔΔG mut-wₑ₅ is the difference in the free energy of binding between the wild-type and mutant cytochrome P450 with either cyt b₅ or cyt b₆. The free energy of binding of the mutant cytochrome P450-cyt b₆ complex was determined and subtracted from the free energy of binding of the wild-type complex.

*b Benzphetamine metabolism and the predicted Vₘₕₐₓ of benzphetamine metabolism are in units of nanomoles product/min/nmol cytochrome P450 ± S.D.

*d ND, not determined.

*e These values differ significantly from wild-type values with p < 0.01.
Determination of Binding Site on CYP2B4 for Its Redox Partners

The ability of the mutants to metabolize the model substrate, benzphetamine, is shown in Table V. The results were obtained from experiments where P450 and P450 reductase were both present at a final concentration of 0.16 μM. All of the P450 mutants except M137A, with diminished ability to bind cyt b5 and mutants R422A and R443A, have a 50–85% reduced rate of benzphetamine metabolism compared with wild type (determined by t test analysis). If the mutants with a normal ability to bind cyt b5 are compared as a group to the mutants showing a reduced ability to bind cyt b5, it can be demonstrated that the mutants with the diminished ability to bind cyt b5 have a significantly lower rate of benzphetamine metabolism than the mutants with normal cyt b5 binding. This finding prompted us to examine the ability of these mutants to bind P450 reductase.

Characterization of the Ability of Wild-type and Mutant P450s to Bind P450 Reductase—A number of previous investigators have identified basic residues on the proximal surface of CYP2B4 which were presumed to participate in binding acidic residues on P450 reductase. Strobel and co-workers (55, 56) demonstrated that when eight lysine residues in CYP2B1, which is 85% identical to CYP2B4, were chemically modified by acetic anhydride, 95% of the activity was lost. Residues that were modified in this inactive protein and their predicted location in the secondary structure of P450 are Lys-384 (β1–3; side chain hydrogen bond to carbonyl of Asp-374 in β2–1), Arg-422 (m-β), Lys-433 (β bulge), and Arg-473 (substrate recognition site 6). These residues correspond to the same residues in CYP2B4. (See Table IV for location of the residues in the secondary structure of P450, as well as the hydrogen bonding pattern of the Arg-422 and Lys-433 side chains.) Fujii-Kuriyama and co-workers (54) mutated the conserved lysine residues in cytochrome P450 1A2 (P450a) by site-directed mutagenesis and showed that several had a decreased ability (2–4-fold) to bind and be reduced by P450 reductase. Some of these residues are predicted to be on the proximal surface of CYP2B4 and correspond to residues Arg-422, Lys-433, and Arg-443 in CYP2B4 which were mutated in the experiments described here. Cyt P450soc (CYP 11A1), a mitochondrial P450, was also mutated at residues Arg-377 and Arg-381 which are predicted to be located in the K helix and correspond to CYP2B4 residues Asp-350 and His-354 (61). Arg-377 and Arg-381 were shown to play crucial roles in binding adrenodoxin. Thus, the consensus of a large body of experimental evidence is that basic residues on the proximal surface of P450 are involved in binding acidic residues on a redox partner. Until recently, our understanding of the result of such mutagenesis studies has been hindered by the unavailability of reliable models of microsomal P450s.

The P450-catalyzed oxidation of substrates requires the transfer of two electrons from P450 reductase. Electron transfer occurs within a transient intermolecular complex between P450 and P450 reductase (33). If complex formation between electron donor and acceptor molecules did not occur, efficient electron transfer would be impossible and electrons would be dissipated into the medium. It has been assumed that the rate of substrate oxidation is proportional to the concentration of the P450-P450 reductase complex, which is an approach similar to that used by Miwa et al. (33). The observation that oxyferrous cytochrome P450 accumulates during turnover (62, 63) supports this assumption, since it indicates that product formation is limited by electron transfer. This relationship can be described by Equation 1.

rate = k[P450-P450 reductase]  
(Eq. 1)

that these experiments were performed at protein concentrations close to the Kd of the P450-cyt b5 complex.

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(Eq. 1)

that these experiments were performed at protein concentrations close to the Kd of the P450-cyt b5 complex.
where rate refers to the rate of substrate oxidation, \( k \) is a proportionality constant, and \([P450-P450 \text{ reductase}]\) is the concentration of the interprotein complex. A general equation that relates the equilibrium dissociation constant of the P450-P450 reductase complex and the rate of substrate oxidation was derived (see “Appendix”) and has been used to determine the \( K_d \) equilibrium dissociation constant, of the P450-P450 reductase complex. The rate of oxidation of the substrate, benzphetamine, was measured experimentally in the presence of varying but known concentrations of P450 and P450 reductase. These data were fit to Equation 21 under the “Appendix,” and the \( K_d \) values between the individual mutant P450s and the reductase were calculated using nonlinear regression.

The apparent \( K_d \) for the wild-type CYTB4-P450 reductase complex is 0.02 ± 0.02 \( \mu \text{M} \), in agreement with previously published values (33). Consistent with the determination of the \( K_d \) values of the individual complexes was the finding that substrate oxidation was proportional to reductase concentration with a fixed concentration of P450. As predicted the weaker binding P450 mutants exhibited a greater percentage increase in product formation with a given increment of the reductase. In the absence of P450 the control reactions exhibited no substrate oxidation. Nine mutant P450s exhibited an increased apparent \( K_d \) in complex with P450 reductase (Table V). The seven mutants, R122A, F135A, M137A, K139A, K433A, R126A, and R133A, that bound cyt \( b_5 \) poorly also bound reductase poorly. In addition, the mutants R422A and R443A showed elevated \( K_d \) values. The binding of these nine mutant P450s to P450 reductase was diminished 6–47-fold (Table V). The location of the mutated amino acids on the surface of CYTB4 that exhibited diminished binding to reductase is shown in Fig. 6B. As mentioned previously, six of the mutants with decreased ability to bind cyt \( b_5 \) are located on the proximal surface of CYTB4 slightly below the heme in the C and C” helices. The location of the other amino acids in the secondary structure of the CYTB4 model and their possible role in its structural stability follows. Lys-433 is in the highly conserved \( \beta \)-bulge three residues away from the heme ligand, cysteine 436. A nitrogen in its side chain forms a salt bridge with the carboxyl group of Asp-90 in the B helix; the remainder of the guanidinium group is exposed to solvent. R422A is in a loop in a conserved region between the meander and the \( \beta \)-bulge above the heme. One of the nitrogens in its side chain guanidinium group forms a salt bridge with the side chain carboxyl of Glu-424 also in the conserved region between the meander and \( \beta \)-bulge. The remainder of the Arg-422 guanidinium group is on the surface exposed to water. Arg-443 is in the L helix, and its side chain forms a salt bridge with the side chain carboxyl group of Glu-439 also in the L helix. Arg-443 is homologous to the P450 camphor residue, Arg-364, which forms a structurally important salt bridge to Glu-286 in the K helix (64) and was predicted to form a salt bridge with P450 camphor’s redox partners, cyt \( b_5 \) and putidaredoxin (14). Table IV lists the hydrogen bonding pattern of the mutated amino acid side chains and the expected structural effects of mutating the amino acid to an alanine. In many instances, structural perturbations are expected, and cavities are created which will give water access to previously shielded surfaces. Our results unexpectedly reveal that the binding sites for cyt \( b_5 \) and P450 reductase have considerable overlap. These results are not in agreement with previous experiments with a purified, covalently cross-linked, functional CYTB4-cyt \( b_5 \) complex (65). The previous studies indicated that there was no overlap between the cyt \( b_5 \) and P450 reductase-binding site on CYTB4. At present, there is no explanation for the discrepancy.

Note that the initial assays for methoxyflurane and benzphetamine metabolism were conducted at total P450 reductase concentrations of 0.2 \( \mu \text{M} \) and 0.16 \( \mu \text{M} \), respectively. The reductase concentrations are 8- and 6.4-fold higher than the \( K_d \) of the P450-P450 reductase complex. The presence of these relatively high concentrations of reductase during the assays of substrate metabolism presumably accounts for the fact that substrate metabolism was only minimally disturbed in those mutants exhibiting decreased ability to bind both P450 reductase and cyt \( b_5 \).

Although the predicted \( V_{\text{max}} \) values for benzphetamine metabolism had a large standard deviation, they were all indistinguishable from the wild-type protein, except for the R126A mutant with a 66% decrease \( V_{\text{max}} \) (Table IV), indicating that in the presence of elevated levels of P450 reductase the mutant P450s function normally.

Characterization of the Free Energy of Binding between the Mutant P450s and Their Redox Partners—The specific interactions between CYTB4 and its redox partners cyt \( b_5 \) and P450 reductase are critical events during substrate oxidation in \textit{vivo} and \textit{in vitro}. In this article, seven cyt P450 residues that participate in the cyt \( b_5 \)-P450 interaction and nine cyt P450 residues that participate in the P450 reductase-P450 interaction have been identified, partially on the basis of the complementarity of the surfaces of cyt \( b_5 \) and a CYTB4 model. In an effort to more thoroughly understand the function of the mutated amino acids in the protein-protein association, the difference in the free energy of binding between the wild-type and mutant P450s and cyt \( b_5 \) was calculated (Table V). The free energy of binding (calculated using the formula \( \Delta G = RT\ln K_d \)) between cyt \( b_5 \) and wild-type CYTB4 is 9.2 kcal/mol. Of the seven mutant proteins with decreased ability to bind cyt \( b_5 \), the R133A mutant protein had the greatest reduction in affinity (>2.2 kcal/mol). The sum of the difference in free energy of binding between the seven mutant P450s and cyt \( b_5 \) was 10.8 kcal/mol, which exceeds the known binding free energy for the entire complex by 1.6 kcal/mol (Table V) (66, 67).

Modeling of the effects of deleting the hydrophobic side chains, hydrogen bonds, and salt bridges, formed by the mutated side chains in CYTB4, indicates that the mutations are likely to lead to local structural and electrostatic perturbations that would contribute to the decrease in the binding of the mutant P450s to cyt \( b_5 \) (Tables IV and V). At least one of the hydrogens of the basic side chains of Arg-122, Arg-133, Lys-139, and Lys-433 interact with an oppositely charged residue in the CYTB4 model and is likely to generate a local structural change when mutated.

Mutation of basic residues on the proximal surface of CYTB4 may also disrupt the molecular dipole which has been suggested to function pre-collisionally, both to orient the interaction of P450 with its redox partners and to promote the electrochemical flow of the reactants involved in oxidation (3). The molecular dipole of P450 is oriented so that it will promote the proximal-to-distal flow of electrons from the redox partner and the distal-to-proximal flow of protons from the solvent.

Previous studies of the cyt \( b_5 \)-cyt \( c \) and the growth hormone-growth hormone receptor interprotein-binding sites indicate that their surfaces are complementary and that a few hydrophobic residues near the center of the interprotein-binding site provide the majority of the binding energy. These core residues were surrounded by less energetically important, more hydrophilic and charged contact residues. Examination of the surface location of the mutated residues, which result in diminished binding of cyt \( b_5 \), shows that Arg-133 (Table IV) appears to be located in the middle of the putative cyt \( b_5 \)-binding site, along with the uncharged amino acids Phe-135 and Met-137. Since a model of the binding site on CYTB4 for cyt \( b_5 \) rather than a
crystal structure of the interprotein complex is being examined, there is insufficient data at this time to allow us to conclude whether or not the CYP2B4-cyt b5-binding site has a cross-section similar to the two other interprotein complexes mentioned previously (66–69).

The free energy of binding between wild-type CYP2B4 and P450 reductase is \(-10.5\) kcal/mol. CYP2B4 binds reductase about 10-fold tighter than it binds cyt b5. The sum of the difference in the free energy of binding between P450 reductase and the nine mutant P450s is \(-14.4\) kcal/mol which exceeds the known binding free energy for the P450-P450 reductase complex (Table V) (67–69). The side chains of Arg-422 (Arg-422 aligns one residue away from Arg-344 on P450 camphor) and Arg-443 (Arg-443 aligns with the structurally important Arg-364 in P450 camphor (64)), which are involved in reductase but not cyt b5 binding, form salt bridges on the surface of the CYP2B4 model with glutamic acids residues 424 and 439, respectively. It should be noted that Sligar and co-workers (14) CYP2B4 model with glutamic acids residues 424 and 439, respectively. It should be noted that Sligar and co-workers (14) predicted that residues Arg-344 and Arg-364 in P450 reductase-CYP2B4 complex, or glutamic acids residues 424 and 439, respectively. It should be noted that Sligar and co-workers (14) predicted that residues Arg-344 and Arg-364 in P450 reductase would be involved in binding cyt b5 and putidaredoxin. The mutation of these charged residues may give rise to local structural perturbations which may account for some of the decrease in binding energy in a given mutant (Table IV).

In summary, the existence of a model of CYP2B4 has enabled us to formulate specific hypotheses aboutting the structural and electrostatic consequences of mutations in basic and neutral amino acids on the proximal surface of CYP2B4. The heme has its closest approach to the surface of the molecule on its proximal face and is the logical place for docking of its redox partners and subsequent electron transfer. The studies reported herein demonstrate that the binding sites on the proximal surface of CYP2B4 for cyt b5 and P450 reductase partially overlap. Basic and neutral residues in the C and C* helices of amino acids on the proximal surface of CYP2B4. The heme has its closest approach to the surface of the molecule on its proximal face and is the logical place for docking of its redox partners and subsequent electron transfer. The studies reported herein demonstrate that the binding sites on the proximal surface of CYP2B4 for cyt b5 and P450 reductase partially overlap. Basic and neutral residues in the C and C* helices of CYP2B4 play an especially prominent role in binding its redox partners.

**Acknowledgments**—We gratefully acknowledge the expert assistance of Thomas Renner and John Rukkila in the preparation of this manuscript.

**APPENDIX**

In the following analysis, an equation is derived that relates the equilibrium dissociation constant of the cyt b5-CYP2B4 complex to the observed type I spectral shift (decrease in absorbance at 420 nm and increase at 385 nm) that occurs when cyt b5 and CYP2B4 are mixed in the presence of the substrate methoxyflurane. This derivation assumes that cyt b5 and CYP2B4 interact in a 1:1 ratio. The dissociation can be described by Equation 2.

\[
P450\text{-}cyt\ b5\ complex \leftrightarrow P450 + cyt\ b5 \quad \text{(Eq. 2)}
\]

The equilibrium constant for the dissociation of the cyt b5-CYP2B4 complex, or \(K_d\), is given in Equation 3.

\[
K_d = \frac{[P][B]}{[PB]} \quad \text{(Eq. 3)}
\]

where \(K_d\) is the equilibrium dissociation constant for the cyt b5-CYP2B4 complex; [P] is the concentration of free P450; [B] is the concentration of free cyt b5; and [PB] is the concentration of the complex. However,

\[
[P] = [P_0] - [PB] \quad \text{(Eq. 4)}
\]

\[
[B] = [B_0] - [PB] \quad \text{(Eq. 5)}
\]

where \([P_0]\) is the concentration of CYP2B4 added and \([B_0]\) is the concentration of cyt b5 added to the reaction mixture. Substituting Equations 4 and 5 above into Equation 6 gives the following:

\[
K_d = \frac{([P_0] - [PB])([B_0] - [PB])}{[PB]} \quad \text{(Eq. 6)}
\]

Rearranging Equation 6 produces Equation 7 as follows:

\[
[PB]^2 - ([PB] + [P_0] + [B_0]) - [P_0][B_0] = 0 \quad \text{(Eq. 7)}
\]

Completing the square yields Equation 8 as follows:

\[
\left(\frac{[PB] - ([K_d + [P_0] + [B_0])}{2}\right)^2 = -[P_0][B_0] + \left(K_d + [P_0] + [B_0]\right)^2 \quad \text{(Eq. 8)}
\]

Taking the square root and solving for [PB] gives Equation 9 as follows:

\[
[PB] = \frac{[K_d + [P_0] + [B_0]}{2} - \sqrt{\frac{[K_d + [P_0] + [B_0]^2}{4} - [P_0][B_0]} \quad \text{(Eq. 9)}
\]

This equation describes the concentration of the CYP2B4-cyt b5 complex in solution for a given \([P_0], [B_0]\), and \(K_d\). The observed absorbance change, \(\Delta A\), which occurs on mixing of cyt b5 and P450, can be derived by Equation 10.

\[
\Delta A = \Delta A_{\text{max}}[PB] \quad \text{(Eq. 10)}
\]

where \(\Delta A_{\text{max}}\) is the maximal absorbance change per unit concentration of P450 (-\((A_{420\text{ initial}} - A_{420\text{ final}})\) - \((A_{385\text{ initial}} - A_{385\text{ final}})\)) occurring when cyt b5 is added to CYP2B4 in the presence of a saturated solution of methoxyflurane. Substituting Equation 9 into Equation 10 gives Equation 11 as follows:

\[
\Delta A = \Delta A_{\text{max}} \left[\frac{[K_d + [P_0] + [B_0]}{2} - \sqrt{\frac{[K_d + [P_0] + [B_0]^2}{4} - [P_0][B_0]} \right] \quad \text{(Eq. 11)}
\]

A similar analysis can be performed to derive an equation that relates the equilibrium dissociation constant of the P450 reductase-CYP2B4 complex (assuming a stoichiometry of 1:1) to the observed rate of formaldehyde production at different reductase concentrations. The dissociation can be described by Equation 12.

CYP2B4-P450 reductase complex \(\leftrightarrow\) CYP2B4 + P450 reductase \quad \text{(Eq. 12)}

The equilibrium dissociation constant of the P450 reductase-CYP2B4 complex, or \(K_d\), is given in Equation 13.

\[
K_d = \frac{[P][R]}{[PR]} \quad \text{(Eq. 13)}
\]

where \(K_d\) is the equilibrium dissociation constant of the P450 reductase-CYP2B4 complex; [P] is the concentration of P450 in solution; [R] is the concentration of P450 reductase in solution; and [PR] is the concentration of the complex. However,

\[
[P] = [P_0] - [PR] \quad \text{(Eq. 14)}
\]

\[
[R] = [R_0] - [PR] \quad \text{(Eq. 15)}
\]

where \([P_0]\) is the total concentration of CYP2B4 and \([R_0]\) is the total concentration of P450 reductase added to the reaction mixture. Substituting Equations 14 and 15 above into Equation 13 gives Equation 16.

\[
K_d = \frac{([P_0] - [PR])([R_0] - [PR])}{[PR]} \quad \text{(Eq. 16)}
\]

Rearranging Equation 16 gives Equation 17.

\[
[PR]^2 - ([PR] + [P_0] + [R_0]) - [P_0][R_0] = 0 \quad \text{(Eq. 17)}
\]
Completing the square gives Equation 18.

\[
\left( [PR] - \left( \frac{K_d + [P_0] + [R_0]}{2} \right) \right)^2 = -\frac{[P_0][R_0]}{4} + \left( \frac{K_d + [P_0] + [R_0]}{2} \right)^2
\]  

(Eq. 18)

Taking the square root and solving for [PR] gives Equation 19.

\[
[PR] = \frac{K_d + [P_0] + [R_0]}{2} \pm \sqrt{\frac{K_d + [P_0] + [R_0]}{2}^2 - \frac{[P_0][R_0]}{4}}
\]

(Eq. 19)

This equation can be used to calculate the concentration of the CYP2B4-P450 reductase complex, in solution for a given [P_0] and [R_0], provided its K_d is known. We have assumed that the rate of formaldehyde production (nmol of product/min/nmol P450) is proportional to [PR] as shown in Equation 20.

\[
rate = V_{\text{max}} [PR]
\]

(Eq. 20)

where V_{\text{max}} is the maximum rate of product formation (nmol of product/min/nmol P450). V_{\text{max}} is a constant for a given CYP2B4 protein and is expected to be constant for all mutant proteins. It assumes an unaltered substrate-binding site, an active site, and an electron transfer process.

By substituting Equation 20 into Equation 19, Equation 21 is produced.

\[
rate = V_{\text{max}} \left[ \frac{K_d + [P_0] + [R_0]}{2} - \sqrt{\frac{K_d + [P_0] + [R_0]}{2}^2 - \frac{[P_0][R_0]}{4}} \right].
\]

(Eq. 21)

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