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Site-directed Mutagenesis of Putative Substrate Recognition Sites in Cytochrome P450 2B11: Importance of Amino Acid Residues 114, 290, and 363 for Substrate Specificity

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SUMMARY

Eleven amino acid residues unique to dog cytochrome P450 (P450) 2B11, compared with rat 2B1 and 2B2, rabbit 2B4 and 2B5, and mouse 2B10, in the putative substrate recognition sites [*J. Biol. Chem.* **267**:83–90 (1992)] were mutated to the residues found in 2B1 or 2B5. The mutants were expressed initially in COS cells and screened for activity toward androstenedione and 2,2′,4,4′,5,5′-hexachlorobiphenyl (245-HCB). P450 2B11 mutants V107I, M199L-N200E-V204R, V234I, A292L, Q473R, and I475S showed no differences from wild-type P450 2B11 in metabolite profiles with either substrate. Mutants V114I, D290I, and L363V exhibited altered androstenedione metabolite profiles and were expressed in *Escherichia coli* for further study with androstenedione, testosterone, 7-ethoxycoumarin, (*R*)- and (*S*)-warfarin, and 245-HCB. With V114I, hydroxylation of steroids and warfarin and 2-hydroxylation of 245-HCB were decreased, whereas 7-ethoxycoumarin *O*-dealkylation and 3-hydroxylation of 245-HCB were unaltered. For D290I, activities toward all substrates were decreased, except for 16β-hydroxylation of testosterone. The activity of L363V was increased 5–6-fold for 16α-hydroxylation of androstenedione and testosterone but was decreased to 40–50% of wild-type activity with 7-ethoxycoumarin and warfarin and to 6–8% of control for 2-hydroxylation of 245-HCB. Alignment of P450 2B11 with P450 101 and superimposition of the 11 mutated 2B11 residues on a P450 101 three-dimensional model suggest that only residues 114, 290, and 363 represent substrate contact residues, in excellent agreement with the experimental results. The data indicate the importance of the three residues 114, 290, and 363 in substrate specificity and regio- and stereoselectivity of P450 2B11 and also demonstrate that the effects of the mutations vary considerably with different substrates.

The P450 superfamily of enzymes is responsible for the oxidation of a wide range of drugs, carcinogens, environmental pollutants, and other xenobiotics in species ranging from humans to bacteria. Any given P450 enzyme usually has the ability to metabolize a number of different substrates, and different P450s exhibit overlapping specificities (1). Despite this versatility, P450 isoforms often show strict regio- and stereospecificity toward some substrates, such as steroids (2). Considerable interest has been focused in recent years on elucidating the structural determinants of catalytic specificity of these enzymes. Several approaches have been adopted, including the construction of chimeric enzymes, the analysis of differences between closely related P450s and between allelic variants of the same P450, and site-directed mutagenesis. In 1992, Gotoh (3) proposed the existence of six SRSs for the P450 2 family, based on alignments of 51 P450 2 sequences and eight bacterial sequences including P450 101 ($P450_{cam}$). All amino acid residues and chimeric fragments identified to date as being critical for substrate specificity of the P450 2 forms fall within or near the putative SRSs. Key amino acid residues include residues 117, 209, and 365 in mouse P450 2A4 and 2A5 (4–6), residues 114, 206, 302, 363, 367, and 478 in rat P450 2B1 (7–12), residue 113 in rabbit P450 2C1, 2C2, 2C4, and 2C5 (13–15), residue 301 in rabbit P450 2C2 (16), residue 364 in rabbit P450 2C3 (17), residue 359 in human P450 2C9 (18), and residue 303 in rabbit P450 2E1 (19).

Identifying the structural determinants of substrate specificity of rat P450 2B1, rabbit P450 2B4 and 2B5, and dog P450

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ABBREVIATIONS: P450, cytochrome P450; SRS, substrate recognition site; 245-HCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl; 2-OHPenCB, 2-OH-2′,4,4′,5,5′-pentachlorobiphenyl; 2-OHHexCB, 2-OH-2′,3,4,4′,5,5′-hexachlorobiphenyl; 3-OHHexCB, 3-OH-2,2′,4,4′,5,5′-hexachlorobiphenyl; HPLC, high performance liquid chromatography; DLPC, dilauroyl-L-3-phosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLC, thin layer chromatography.

2B11[3] has been a major interest of this laboratory in recent years. These enzymes hydroxylate androstenedione and testosterone, with characteristic metabolite profiles for each form. Of particular interest is the unique ability of dog P450 2B11 to metabolize the polychlorinated biphenyl 245-HCB (20, 21). Investigations into the structural basis for this activity began with the construction of 2B11–2B5 hybrid enzymes (22). Functional studies with eight chimeras provided evidence for two regions of P450 2B11 and 2B5 (amino acid residues 95–239 and 240–370) that appear to be involved in defining regio- and stereoselectivity for androstenedione and three regions of P450 2B11 (residues 95–239, 240–370, and 371–494) that are necessary for 245-HCB metabolism.

The deduced amino acid sequence of dog P450 2B11 exhibits 75% identity with rat P450 2B1, 74% identity with rat P450 2B2, 79% identity with rabbit P450 2B4 and 2B5, and 75% identity with mouse P450 2B10 (21). The >100 amino acid substitutions between P450 2B11 and its rat, rabbit, and mouse counterparts present a formidable challenge to understanding the molecular basis for 245-HCB hydroxylase activity. However, comparison of the amino acid sequences of the putative SRSs of the six 2B enzymes reveals only 11 residues that are unique to the dog enzyme. We hypothesized that critical determinants of the unique 245-HCB hydroxylase activity of P450 2B11 might be found among these residues, and we therefore decided to mutate them to the amino acids present in rat P450 2B1 or rabbit P450 2B5. In a previous report, one such mutant (V114I) was constructed, and evidence from heterologous expression in COS cells showed a 3-fold increase in the ratio of androstenedione 16β- to 16α-hydroxylation (22). In addition, V114I lost the ability to form two of three 245-HCB metabolites, which were at that stage unidentified. The metabolites produced by liver microsomes from a phenobarbital-treated dog have subsequently been identified as 2-OHPenCB, 2-OHHexCB, and 3-OHHexCB (23), and generation of these metabolites by purified hepatic as well as *Escherichia coli*-expressed P450 2B11 has been confirmed in our laboratory.[4] We report now the mutagenesis of 10 additional unique SRS residues in P450 2B11. The metabolism of a number of substrates including 245-HCB has been analyzed, and two new key residues, Asp-290 and Leu-363, have been identified as being important for substrate specificity. The selection of residues for mutagenesis based on the combined criteria of residence in an SRS and being unique in a series of related P450s has provided a powerful approach for identifying determinants of substrate specificity.

Experimental Procedures

Materials. Primers for site-directed mutagenesis and DNA sequencing were obtained from the University of Arizona Macromolecular Structure Facility (Tucson, AZ). Growth media for *E. coli* were obtained from Difco (Detroit, MI). Restriction endonucleases, DNA modification enzymes, and growth media for COS cells were purchased from GIBCO-BRL (Grand Island, NY). Androstenedione, 16α-OH-androstenedione, NADPH, DLPC, chloroquine, dimethylsulfoxide, and all reagents for immunodetection of proteins immobilized on nitrocellulose were purchased from Sigma Chemical Co. (St. Louis, MO). 15α-OH-androstenedione was obtained from D. W. Kirk, University of London (London, England). Other hydroxylated metabolites of androstenedione and testosterone were obtained from Steraloids (Wilton, NH). [4-^{14}C]Androst-4-ene-3,17-dione (androstenedione) and [4-^{14}C]testosterone were purchased from DuPont-New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL). TLC plates [silica gel, 250 μm, Si 250 PA (19c)] were obtained from J.T. Baker Inc. (Phillipsburg, NJ). HEPES buffer was from Calbiochem Corp. (La Jolla, CA). Defined fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). DEAE-dextran was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). COS cells were obtained from Dr. John Regan (University of Arizona, Tucson, AZ). Materials for expression of P450 2B11 in *E. coli* and for assay of 245-HCB metabolism were as described elsewhere.[4]

Site-directed mutagenesis. The U.S.E. mutagenesis kit from Pharmacia (Piscataway, NJ) and the transformer site-directed mutagenesis kit from Clontech Laboratories, Inc. (Palo Alto, CA) were used for the introduction of specific base changes into the P450 2B11 cDNA in the expression vector pBC12BI. The unique restriction enzyme site eliminated in the process of mutagenesis was an *Eco*RV site in the vector close to the 5′-end of the cDNA. Nine mutagenic oligonucleotide primers were synthesized, and their sequences are shown in Table 1. All mutated codons were confirmed by double-stranded sequencing using a Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, OH).

Heterologous expression. COS cells were grown and transfected and COS cell microsomes were prepared 72 hr after transfection as described previously (8), including a washing step (9). Selected mutants were subcloned from the pBC12BI vector into pKKm66c for expression in Topp 3 cells (Stratagene, La Jolla, CA). pKKm66c represents a 2B11 cDNA modified at the 5′ end for expression in *E. coli*, according to the method of Barnes *et al.* (24), and subcloned into the vector pKK233–2 (Pharmacia).[4] The modification results in five amino acid substitutions, Glu-2 to alanine, Ser-4 to leucine, Val-5 to leucine, Leu-6 to alanine, and Leu-7 to valine, but does not affect the regio- or stereospecificity of androstenedione or 245-HCB metabolism[4] or of testosterone or warfarin metabolism (see below). Subcloning of mutants was accomplished by excision of an 832-base pair fragment of 2B11 from the pBC12BI vector by digestion with *Stu*I and *Bst*EII and replacement of the corresponding fragment from pKKm66c. Mutated codons 114, 290, and 363 in pKKm66c were confirmed by sequencing as described above. Growth conditions for expression of the mutants and preparation of a solubilized membrane fraction for activity studies will be described elsewhere.[4]

Incubation conditions. Experiments with COS microsomes were performed as described previously (8–12). All incubations involving *E. coli*-expressed P450 included 10–100 pmol of P450, 2 nmol of rat liver NADPH-P450 reductase/nmol of P450, 30 μg/ml DLPC, 2 nmol of rat

TABLE 1

Nucleotide sequences of P450 2B11 mutagenic primers
Base changes are denoted by underlining

Primer	Sequence
V107I	GTAGTGGAGCCAATCTTCCAGGG
M199L-N200E-V204R	GAGTTCCTGCGCCTGCTGGAGTTGT-TCTATCGGTCCTTCGCACTCATC
V234I	CACAGGCAGATCTACAATAAC
D290I	AACCTCATAATCACGGCGCTC
A292L	CTCATAGACACGCTGCTCTCGCTCTTC
L363V	GGGGACCTTGTCCCCATTGGC
Q473R	TCACACCCCGGGAGATAGGTG
I475S	CCAGGAGAGTGGTGTGGGCAA

[3] The P450 2B gene subfamily is composed of P450s from different species that have been grouped together on the basis of amino acid sequence identity, in accordance with the suggested nomenclature (35). This study deals with site-directed mutants of P450 2B11 differing from the wild-type enzyme at residues 107, 114, 199/200/204, 234, 290, 292, 363, 473, and 475. The wild-type enzyme is referred to as P450 2B11 and has the deduced amino acid sequence defined by Graves *et al.* (21) (GenBank accession numbers M33575 and M92447). Nine site-directed mutants differing in 11 amino acid residues are described in the current study. Mutants are indicated using the single-letter code for the amino acid replaced, the position in the sequence, and the designation of the new residue, in that order (36). For example, V107I refers to replacement of valine at position 107 by isoleucine.

[4] G. H. John, J. A. Hasler, Y.-A. He, and J. R. Halpert. *E. coli* expression and characterization of cytochromes P450 2B11, 2B1, and 2B5. Submitted for publication.

liver cytochrome b_5/nmol of P450, 2 mM NADPH, and the relevant substrate, in 50 mM HEPES, pH 7.6, 15 mM $MgCl_2$, 0.1 mM EDTA. The reaction was set up as follows: P450, NADPH-P450 reductase, 30 μg/ml DLPC, and buffer in one fourth of the final incubation volume were mixed and incubated for 10 min at room temperature. A buffer mixture containing DLPC (30 μg/ml) and cytochrome b_5 was prepared and added to the reconstituted enzymes. Substrate was added, and the sample was vortexed for 30–40 sec and preincubated for 3 min at 37°. NADPH was added, and the mixture was incubated at 37° for the appropriate time.

Steroid hydroxylase assays. The assays of androstenedione hydroxylase activity (8, 25) and testosterone hydroxylase activity (26) were performed as described previously. Metabolites were resolved on TLC plates by two cycles of chromatography in ethyl acetate/chloroform (2:1, v/v) for 16β-OH- and 16α-OH-androstenedione and in dichloromethane/acetone (4:1, v/v) for 15α-OH- and 6β-OH-androstenedione and for testosterone metabolite separation.

7-Ethoxycoumarin deethylase assay. Activity was determined fluorimetrically using an excitation wavelength of 366 nm and an emission wavelength of 454 nm. The deethylation reaction was stopped by the addition of 2 N HCl and metabolites were extracted with chloroform and back-extracted with 30 mM sodium borate (27).

Warfarin hydroxylase assay. Incubations with (*R*)- and (*S*)-warfarin and identification and quantification of metabolites were as described previously (28).

245-HCB hydroxylase assay. The 1-ml reaction was terminated by acidification to pH 3.0 with 60 μl of 1 N H_2SO_4 and was analyzed by reverse phase chromatography (22). The hydroxylated 245-HCB metabolites were extracted three times with 3 ml of HPLC-grade ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness under a stream of nitrogen at room temperature. The dried residue was redissolved in HPLC-grade acetonitrile and transferred to a 1.5-ml microfuge tube. The redissolved mixture was evaporated to 35 μl, centrifuged a final time to sediment any particulates, and injected into the HPLC system for analysis. The chromatography conditions were as follows: Brownlee RP-8 (Rainin) guard column, Zorbax RX-C8 4.6-mm (i.d.) × 25-cm column, 1.5 ml/min flow rate, and 58% component B (0.05%, w/v, trifluoroacetic acid in filtered acetonitrile)/42% component A (0.1%, w/v, trifluoroacetic acid in filtered Millipore water) mobile phase. Detection was by UV absorbance at 220 nm. Three major metabolites eluted at approximately 17, 20, and 24 min, representing 2-OHPenCB, 3-OHHexCB, and 2-OHHexCB, respectively. The peak area from the HPLC chromatogram was shown to be proportional to the amount of metabolite generated through the use of radiolabeled 245-HCB. Details of the identification by gas chromatography-mass spectrometry or coelution with authentic standards of the metabolites produced by wild-type P450 2B11 will be presented elsewhere.[4]

Immunoblot analysis. Separation of proteins on sodium dodecyl sulfate-polyacrylamide gels (7.5%) (29), transfer of proteins to nitrocellulose, and immunodetection of P450 proteins were carried out as described previously (8, 30).

Homology alignment and graphic analysis. The amino acid sequence of P450 2B11 was obtained from the SwissProt database (accession number P24460) (21). The alignment of P450 2B11 with P450 101 (accession number P00183) was performed as described elsewhere.[5] Briefly, the sequences were initially aligned using the PILEUP and GAP programs from the GCG suite of programs (31). The gaps introduced in structurally conserved regions (especially helices) were removed manually using the INSIGHTII/HOMOLOGY package (Biosym Technologies, San Diego, CA). The secondary structure prediction with the profile network (PHD) method (32), as well as the Dayhoff mutation matrix and the Engleman and Steitz hydrophobicity index, was used to determine structurally conserved regions. This established a correspondence between our mutated residues and appropriate amino acid residues of P450 101. According to this alignment, certain residues in the three-dimensional structure of P450 101 were replaced with residues of P450 2B11 using the BIOPOLYMER program (Biosym Technologies). The crystal coordinates of P450 101 were obtained from the Brookhaven Protein Databank (33), and the structures were displayed with the INSIGHTII program on a Silicon Graphics workstation.

[5] G. D. Szklarz, R. L. Ornstein, and J. R. Halpert. Application of 3-dimensional homology modeling of cytochrome P450 2B1 for interpretation of site-directed mutagenesis results. Submitted for publication.

Results

Expression in COS cells. Mutant P450 2B11 forms were constructed using the mutagenic primers shown in Table 1. The amino acids present in 2B11 were mutated to the corresponding amino acid found in rat 2B1 or rabbit 2B5. Because previous results with a P450 2B11–2B5 hybrid suggested the potential importance of SRS-6 for 245-HCB metabolism by 2B11 (22), residues 473 and 475 in 2B11 were mutated to the 2B5 residue. However, the greater identity between 2B11 and 2B1 than between 2B11 and 2B5 at key sites in the other SRSs prompted us to mutate the other nine unique 2B11 SRS residues to those found in 2B1. Because residue 475 is identical in 2B1 and 2B5, the net result of these considerations is that in 10 of 11 positions P450 2B11 was mutated to P450 2B1. Three of the amino acid residues of interest (Met-199, Asn-200, and Val-204) were adjacent to each other and were mutated together to form a triple mutant. The effects of the mutations in the SRSs were screened initially by expression in COS cells. Expression levels in COS cell microsomes were assessed by immunoblotting, and function was assayed with androstenedione. The immunoblots (Fig. 1A) show comparable expression levels for wild-type 2B11 and all mutants except for D290I (Fig. 1A, *lane 6*), which gave low levels of expression in two transfection experiments. The mutant V114I constructed previously was also not well expressed in COS cells (data not shown). Of the new mutants, only D290I and L363V exhibited marked alterations in androstenedione hydroxylase profiles (Fig. 1B; Table 2). Assays of 245-HCB metabolism by COS microsomal preparations indicated that, except for V114I, D290I, and L363V, all mutants yielded the same metabolite profile as did the wild-type enzyme (data not shown). In contrast, it was not possible to detect 245-HCB metabolism by any of the mutants V114I, D290I, and L363V. This was not surprising for V114I and D290I, because expression levels in COS cells were low.

Expression in *E. coli*. V114I, D290I, and L363V were expressed in *E. coli* to study the effects of mutations at these sites on metabolism of a number of substrates including 245-HCB. The results obtained with androstenedione and testosterone are shown in Table 3, with Fig. 2 illustrating a typical autoradiogram obtained for androstenedione. V114I and D290I exhibited marked decreases in androstenedione 16β- and 16α-hydroxylase and testosterone 16α-hydroxylase activities, whereas testosterone 16β-hydroxylase activity was within ± 50% of the wild-type 2B11 activity. The ratios of androstenedione 16β:16α hydroxylation for V114I and D290I were 4:1 and 9:1, respectively, compared with a value of 1:1 for the wild-type enzyme. For rat P450 2B1, the ratio is 7–8:1 (8–12). Thus, both the Val-114 to isoleucine and Asp-290 to isoleucine substitutions, which exchange the 2B11 residue for the 2B1 residue, make the dog enzyme more like the rat enzyme in androstenedione hydroxylation profiles. L363V exhibited a 5–6-fold increase in 16α-hydroxylase activity toward both steroids as well as acquisition of 15α-hydroxylase activity, which is not detectable in wild-type 2B11.

Fig. 3 shows the profile of 245-HCB metabolites produced by

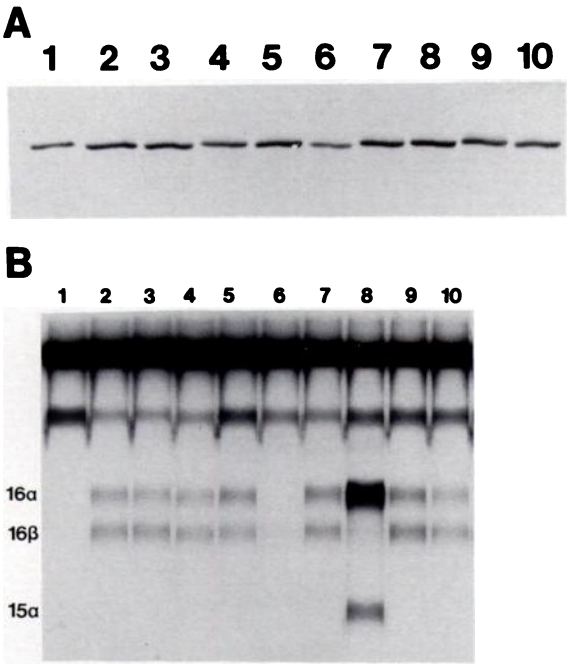

Fig. 1. A, Immunoblot of microsomes prepared from COS cells transfected with wild-type and mutant P450 2B11 cDNAs (*lanes 2-10*). *Lane 1*, purified P450 2B11 protein standard; *lane 2*, wild-type expressed 2B11; *lane 3*, V107L; *lane 4*, M199L-N200E-V204R; *lane 5*, V234I; *lane 6*, D290I; *lane 7*, A292L; *lane 8*, L363V; *lane 9*, Q473R; *lane 10*, I475S. Proteins (20 μg of protein in each case) were separated on a denaturing 7.5% sodium dodecyl sulfate gel and transferred to nitrocellulose. The expressed proteins were detected with rabbit anti-rat P450 2B1 (primary antibody), goat anti-rabbit IgG conjugated to alkaline phosphatase (secondary antibody), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color reagents. B, Autoradiogram of androstenedione metabolites produced by microsomes from COS cells transfected with wild-type and mutant P450 2B11 cDNAs. *Lane 1*, sham transfected; *lane 2*, wild-type 2B11; *lane 3*, V107L; *lane 4*, M199L-N200E-V204R; *lane 5*, V234I; *lane 6*, D290I; *lane 7*, A292L; *lane 8*, L363V; *lane 9*, Q473R; *lane 10*, I475S. Microsomal protein (100 μg) was incubated with 25 μM [^{14}C] androstenedione for 15 min at 37° in 100 μl of buffer containing 20 pmol of NADPH-P450 reductase and 10 pmol of cytochrome b_5. Samples were quenched by the addition of 50 μl of tetrahydrofuran, and 50-μl aliquots were applied to a TLC plate. Metabolites are 16α-OH-, 16β-OH-, and 15α-OH-androstenedione. The data are shown in Table 2.

TABLE 2

Metabolism of androstenedione by wild-type and mutant P450 2B11 expressed in COS cell microsomes

Values for metabolism of androstenedione (AD) are derived from duplicate incubations performed with 50–100 μg of COS cell microsomal protein/assay and are expressed as pmol of metabolite/min/mg of protein, after correction with blanks of control COS microsomes. Ratios represent the means of four incubations.

Wild-type/mutant	SRS[a]	Metabolite formation			
		15α-OH-AD	16β-OH-AD	16α-OH-AD	16β/16α-OH-AD ratio
		pmol/min/mg			
Wild-type		<0.2	36	32	1.1
V107I	1	<0.2	36	32	1.1
M199L-N200E-V204R	2	<0.2	34	31	1.1
V234I	3	<0.2	22	32	0.7
D290I	4	<0.2	4.4	0.4	11
A292L	4	<0.2	33	36	0.9
L363V	5	48	9.2	325	0.03
Q473R	6	<0.2	50	49	1.0
I475S	6	<0.2	50	46	1.1

[a] See Ref. 3.

the wild-type and mutant P450 2B11 forms expressed in *E. coli*. P450 2B11 produces the three metabolites 2-OHPenCB, 3-OHHexCB, and 2-OHHexCB in the ratio 2:1:1.5. All three mutants retained <10% of the ability to produce the 2-OH metabolites. The V114I mutant, however, showed complete retention of 3-hydroxylase activity, whereas D290I and L363V produced the 3-OH metabolite at about 25–30% of the rate of the wild-type enzyme. These findings are completely consistent with the observation that the 3-OH metabolite can be produced to a limited extent by rats and rabbits and with the hypothesis that both 2-OH metabolites unique to dogs arise from a common intermediate, possibly an epoxide (23). Overall, the data in Fig. 3 suggest that the unique ability of P450 2B11 to metabolize 245-HCB results from a binding orientation that allows 2-hydroxylation and that this is highly dependent on the identity of residues 114, 290, and 363.

Table 4 shows ethoxycoumarin and warfarin metabolism by the wild-type and mutant P450 2B11 expressed in *E. coli*. V114I retained 100% of the 7-ethoxycoumarin deethylase activity of P450 2B11, whereas both D290I and L363V showed decreased activity. With (*R*)-warfarin, all three mutants displayed decreased ability to form the 4′-OH metabolite. 4′-Hydroxylation of (*S*)-warfarin was catalyzed by 2B11 at a rate 10-fold lower than *R*-4′-hydroxylation, and only V114I showed detectable *S*-4′-hydroxylase activity. Interestingly, although rates of warfarin metabolism were decreased with the mutants, there was no evidence of a change in the regioselectivity of (*R*)- or (*S*)-warfarin hydroxylation. This is consistent with the observations that rat 2B1 and 2B11 both metabolize (*R*)-warfarin primarily at the 4′-position and that the dog enzyme is 10-fold more active (20).

Homology alignment and three-dimensional modeling. Lately, homology modeling has been increasingly used to probe structure-function relationships of P450 enzymes, including our recent modeling study of P450 2B1.[5] Therefore, this methodology was applied in an effort to explain the experimental data obtained with the 2B11 mutants and to assess the validity of the model. After sequence alignment of P450 2B11 with P450 101 (Fig. 4), residues in P450 101 were replaced with the residues of P450 2B11 mutated in the current study. Fig. 5 shows a model of P450 101 with these 2B11 residues displayed and viewed down helix I. The model suggests that residues 114, 290, and 363 are close to or part of the substrate binding site where heme and Thr-302 (Thr-252 in P450 101) are located. Conversely, those SRS residues for which mutations had no effects on P450 2B11 activity are far from the binding site. In this context it should be emphasized that the SRSs correspond to P450 101 substrate binding sites extended by three amino acids on both sides (3). Therefore, not all SRS residues would be expected to correspond to substrate contact residues in P450 101. The critical finding of our study is that none of these peripheral SRS residues appears to account for differences in substrate specificity between P450 2B11 and 2B1. The results strongly indicate that homology modeling can provide an excellent complement to the SRS model for directing future mutagenesis studies.

Discussion

In an ongoing study of structure-function relationships in the P450 2B subfamily, we have mutated amino acid residues that are unique to dog P450 2B11, within putative SRSs, to the corresponding residues found in rat P450 2B1 or rabbit 2B5.

TABLE 3

Metabolism of androstenedione and testosterone by P450 2B11 expressed in *E. coli*

Values for androstenedione hydroxylation are means ± standard deviations of assays performed with three different P450 preparations, each in duplicate. Values for testosterone hydroxylation represent means of duplicate incubations with one P450 preparation. All assays were performed with 10 pmol of P450/assay, and data are expressed as nmol of metabolite/min/nmol of P450. Testosterone 6β-hydroxylase activities are not shown and were <0.2 nmol/min/nmol of P450 in all cases.

Wild-type/mutant	Androstenedione hydroxylation				Testosterone hydroxylation		
	16β	16α	15α	16β/16α ratio	16β	16α	15α
	nmol/min/nmol				nmol/min/nmol		
Wild-type	2.8 ± 1.6	2.6 ± 1.5	ND[a]	1.07 ± 0.02	0.9	13.3	<0.1
V114I	0.4 ± 0.05	0.1 ± 0.02	ND	4.2 ± 0.5	0.6	1.6	<0.1
D290I	0.3 ± 0.1	0.04 ± 0.02	ND	9.0 ± 0.3	1.3	1.6	<0.1
L363V	0.8 ± 0.2	11.7 ± 2.1	2.0 ± 0.4	0.06 ± 0.005	0.7	79.9	3.3

[a] ND, not detectable.

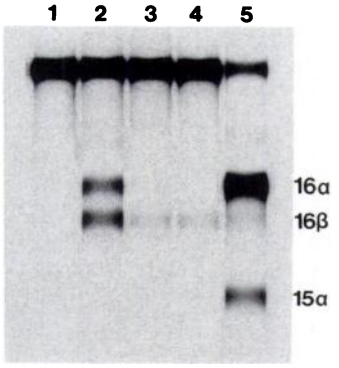

Fig. 2. Autoradiogram of androstenedione metabolites produced by *E. coli*-expressed wild-type and mutant P450 2B11. *Lane 1*, plasmid with no 2B11; *lane 2*, wild-type 2B11; *lane 3*, V114I; *lane 4*, D290I; *lane 5*, L363V. P450 (10 pmol) was incubated with 25 μM [^{14}C]androstenedione for 10 min at 37° in 100 μl of buffer containing 20 pmol of NADPH-P450 reductase, 20 pmol of cytochrome b_5, and 3 μg of DLPC. Samples were quenched by the addition of 50 μl of tetrahydrofuran, and 50-μl aliquots were applied to a TLC plate. Metabolites are 16α-OH-, 16β-OH-, and 15α-OH-androstenedione. The data are shown in Table 3.

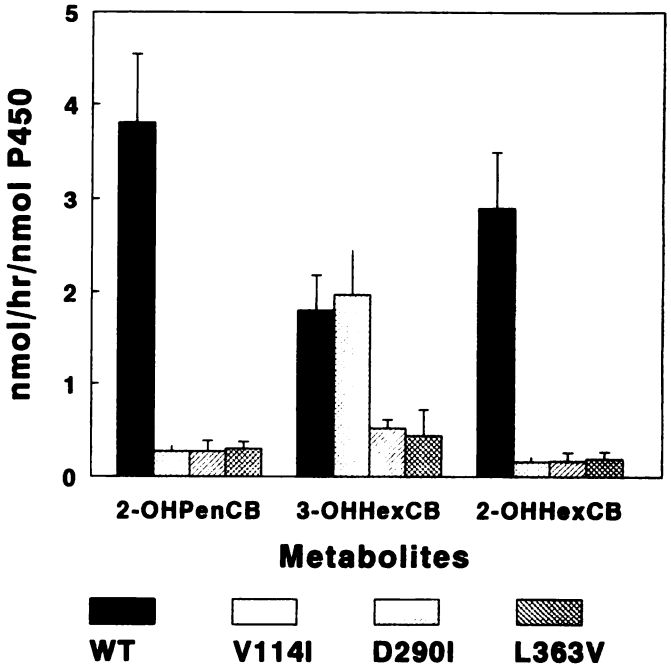

Fig. 3. Profile of metabolites of 245-HCB produced by the soluble membrane fraction from *E. coli* expressing wild-type (*WT*) and mutant P450 2B11. Metabolites are 2-OHPenCB, 3-OHHexCB, and 2-OHHexCB. P450 (100 pmol) was incubated with 60 μM 245-HCB for 1 hr at 37° in 1 ml of buffer containing 200 pmol of NADPH-P450 reductase, 200 pmol of cytochrome b_5, and 30 μg of DLPC. Metabolites were extracted with ethyl acetate and separated by reverse phase HPLC (22).

The rationale for this approach was that the residues occurring at these sites might determine the differences in substrate specificity between dog 2B11 and other 2B isoforms and in particular might account for the unusual ability of 2B11 to metabolize 245-HCB. The 11 amino acids unique to 2B11 represent both conservative and nonconservative amino acid differences, compared with other members of the 2B subfamily. Heterologous expression of the P450 2B11 mutants in COS cells and in *E. coli* demonstrates the importance of residues 114, 290, and 363 for substrate specificity and regio- and stereoselectivity of 2B11. This study also demonstrates that the remaining SRS residues (residues 107, 199, 200, 204, 234, 292, 473, and 475) that are unique to dog P450 2B11 are not likely to play a role in substrate specificity, based on the amino acid substitutions investigated. This does not, however, totally preclude these residues from playing some role in the activity of P450 2B11. Interestingly, alignment of P450 2B11 with P450 101 and superimposition of the 11 mutated 2B11 residues on a P450 101 three-dimensional model indicate that only residues 114, 290, and 363 represent substrate contact residues, in perfect agreement with the experimental results. It should be noted that other residues shown to be important for P450 2B1 (Phe-206, Thr-302, Val-367, and Gly-478) are conserved between 2B1 and 2B11 and may also be important for 2B11 but are unlikely to account for differences between 2B1 and 2B11. Overall, the results suggest that use of the SRS model in conjunction with three-dimensional homology models should

TABLE 4

Metabolism of 7-ethoxycoumarin and warfarin by P450 2B11 expressed in *E. coli*

Values for 7-ethoxycoumarin metabolism are means ± standard deviations of assays performed with three different P450 preparations, each in duplicate, with 10 pmol of P450/assay. Values for warfarin hydroxylase are means of six determinations with one P450 preparation. Data are expressed as nmol of metabolite/min/nmol of P450.

Wild-type/mutant	Metabolism		
	7-Ethoxycoumarin	Warfarin	
		(*R*)-4′-OH	(*S*)-4′-OH
	nmol/min/nmol		
Wild-type	5.8 ± 1.8	13.5	1.4
V114I	5.8 ± 1.4	6.5	0.9
D290I	0.8 ± 0.5	3.0	ND[a]
L363V	2.8 ± 0.7	5.1	ND

[a] ND, not detectable.

```
P450 2B11: melsvllllalltgllllmarghpkayghLPPGPRPLP------ilgnflqmdrkGLLKSFLRLQEky--   62
P450 101 :                                NLAPLPPHVPEHLVFDFDMYNPSNLSAGVQEAWAVLQESNVP   51
                                            (25,139)                  (14,135)

P450 2B11: GDVFTVYLGPRRTVMlcGIDAIREALVDnaEAFSGRGKIAvVEPVFQGYGVVfang--ERWKTLRRFSLA  130
P450 101 : DLVWTRCNGGHWIAT--RGQLIREAYED-YRHFSSECPFI-PREAGEAYDFIPTSMDPPEQRQFRALANQ  117
               (4,107)       (11,128)     (5,135)     (8,128)              (0,111)

P450 2B11: TMRDFGMGKrsVEERIQEEAQCLVEELRKTEGVLQdptffFHSMTANIICSIVFGkrfgykdpeflRLMN  200
P450 101 : VVGMPVVDK--LENRIQELACSLIESLRPQGQCNF--TEDYAEPFPIRIFMLLAGL-------PEEDIPH  176
                           (14,129)                  (1,123)

P450 2B11: LFYVSFALIssfssqmfelfhsflkyfpgthrQVYNNLQEIKAFIARMVEKHRETldpsaprdfiDAYLI  270
P450 101 : LKYLTDQMT-----------------RPDGSMTFAEAKEALYDYLIPIIEQRRQK-------PGTDAISI  222
           (10,126)                                (10,113)                 (7,126)

P450 2B11: RMDkekaepssefHHRNLIDTALSLFFAGTETTSTTLRYGFLLMLKYphiAERIYKEIDqvigphrlpsl  340
P450 101 : VANGQVNGRPI--TSDEAKRMCGLLLVGGLDTVVNFLSFSMEFLAKS---PEHRQELIERPE--------  279
                                  (4,135)                  (7,113)

P450 2B11: ddrakmpYTDAVIHEIQRFGDLLPIGVPHmvTKDICFRGYIIp-KGTEVFPILHSALNDphyfekpdvfn  409
P450 101 : -------RIPAACEELLRRFSLVADGRIL--TSDYEFHGVQLKKGDQILLPQMLSGLDE----------R  330
                       (1,113)              (14,122)      (13,122)

P450 2B11: pdhfldangalkkneafipFSIGKRICLGEGIARMELFLFFTTILQNFsvaspmAPEDIDLTPQEIGVGK  479
P450 101 : ENACPMHVDFSRQKVSHTTFGHGSHLCLGQHLARREIIVTLKEWLTRI---PDFSIAPGAQIQHKSGIVS  397
                                    (20,131)                          (0,125)

P450 2B11: LPPVYQIsflsrggc                                                        494
P450 101 : GVQALPLVWDPATTKAV                                                      414
```

Fig. 4. Alignment of P450 2B11 with P450 101. The mutation matrix score and hydrophobicity score are indicated below structurally conserved regions shown in *capital letters* in the 2B11 sequence. Positive values of a mutation matrix (scale, −80 to 170) and high scores of a hydrophobicity index (scale, 0 to 160) indicate high homology. The residues studied by site-directed mutagenesis are indicated in *bold type*, along with the conserved Thr-302 as a reference point.

provide a powerful means for targeting specific amino acid residues for site-directed mutagenesis studies.

The importance of residues 114 and 363 in 2B11 is consistent with the findings of other studies on the P450 2 family and the 2B subfamily in particular (4–15). For example, based on experimental evidence from site-directed mutagenesis (12) and on molecular modeling of 2B1,[5] it has been proposed that a smaller hydrophobic residue at these positions leads to binding of androstenedione that favors 16α-hydroxylase over 16β-hydroxylase activity, whereas a larger residue favors 16β-hydroxylation. For 2B1, the mutant V363L exhibits enhanced stereospecificity for 16β-hydroxylation but a decrease in total activity (12). The results reported here for the reciprocal mutant L363V of dog 2B11 are fully consistent with the observations on the 2B1 mutant, in that decreasing the size of the residue at position 363 (leucine to valine) favors hydroxylation on the α-face of the steroid and leads to a dramatic increase in total activity. This is the first report, however, of the importance of residue 290 in subfamily 2B. It is interesting to note that this residue is hydrophobic (isoleucine or leucine) in 2B1, 2B4, and 2B5 but polar (aspartate) in 2B11. To date, most substitutions in P450 2B1 that alter substrate specificity involve changes in the size of hydrophobic residues (12). Thus, Asp-290 is the first example of a charged 2B residue of critical significance for substrate specificity. However, it should be emphasized that the substrates examined in the current study are all hydrophobic, and none contains a positive charge, such that charge-pairing between Asp-290 and the substrates is not possible. Interestingly, substitution of Ile-290 in P450 2B1 with aspartate renders the enzyme much more similar to P450 2B11 in terms of the androstenedione hydroxylase profile.[6]

The hypothesis that the changes at positions 114, 290, and 363 affect substrate interactions with the enzyme *per se*, rather than monooxygenase activity in general, is supported by the observation that each mutant exhibits activities comparable to or greater than that of wild-type P450 2B11 with at least one substrate (Table 5). This is readily seen for V114I with ethoxycoumarin deethylase and 245-HCB 3-hydroxylase activities and for L363V with androstenedione and testosterone 16α-hydroxylase activities. For D290I, most activities are decreased except for testosterone 16β-hydroxylase, although this is a minor activity of the wild-type enzyme. The difference in the effects of the mutations on 16β-hydroxylation of androstenedione and testosterone is also noteworthy. It appears that 16β-hydroxylation of testosterone is much less susceptible to the amino acid substitutions than is 16β-hydroxylation of androstenedione (Table 5), with the only difference between the two steroids being a hydroxyl instead of a carbonyl substituent at C-17. Interestingly, L363V exhibits an approximately 5-fold increase in 16α-hydroxylase activity, regardless of whether the substrate is androstenedione or testosterone.

Explanations for the differential effects of the mutations observed with the various substrates (Table 5) are likely to lie in the unique orientations adopted in the active site, although changes in the efficiency of coupling of NADPH utilization to product formation may also play a role (12, 22). Although androstenedione and testosterone are relatively large, rigid,

[6] Y.-a. He, unpublished observations.

Fig. 5. A Cα trace of P450 101 with mutated residues in P450 2B11 displayed, viewed down helix I. *Numbers*, positions in the 2B11 sequence. Heme (*red*) and Thr-302 (*light blue*) are displayed to indicate the location of the active site. The P450 2B11 residues that effected changes in activity upon mutagenesis are shown in *darker blue*. Residues that did not alter activity after mutagenesis are in *orange*. Although residues 473 and 475 may appear to be close to the active site in this two-dimensional projection, from the three-dimensional model or other two-dimensional projections it is evident that these residues are located in that part of β-sheet 5 that is orientated away from the substrate binding site.

planar molecules, ethoxycoumarin, warfarin, and 245-HCB are smaller and less planar. In particular, 245-HCB has a structure in which the *o*-chlorine substituents on each ring repel each other so that the benzene rings do not lie in the same plane. Steroids may fit more tightly into the substrate binding site than do the smaller, less planar substrates but, nevertheless, orientation of substituents of the smaller molecules is probably very important. For example, Table 5 shows that 2-hydroxylation of 245-HCB is more susceptible than 3-hydroxylation to substitutions at positions 114, 290, and 363. The 2-hydroxylation of the first biphenyl ring is likely to be highly sterically hindered by the second ring with its chlorine substituents. The effect of the Val-114 to isoleucine substitution on the various activities is also interesting in light of the recent study by Straub *et al.* (34) of hydrophobic side chain requirements of the corresponding amino acid 113 in P450 2C2 for hydroxylation of two substrates, lauric acid and progesterone. Those results showed that the hydrophobic requirements of this residue are more stringent for a larger, more rigid, steroid substrate than for the saturated fatty acid with a flexible carbon tail. Our results for V114I could be interpreted in the same way if one considered only androstenedione and ethoxycoumarin. By investigating a number of different substrates, however, it is apparent from our data that there is a spectrum of effects of the same mutation on the metabolism of different compounds. Explanations for these observations may come from modeling

TABLE 5

Effect of mutations in P450 2B11 on activity with various substrates, relative to wild-type activity

Data were compiled from Tables 2 and 3 and Fig. 3.

Substrate/metabolite	Metabolism		
	V114I	D290I	L363V
	% of wild-type activity		
Androstenedione			
16α-OH	4	2	450
16β-OH	14	11	29
Testosterone			
16α-OH	12	12	600
16β-OH	67	144	78
7-Ethoxycoumarin	101	14	49
(*R*)-Warfarin	48	22	38
245-HCB			
2-OHPenCB and 2-OHHexCB	6–7	7	6–8
3-OHHexCB	108	29	24

of P450 2B11 together with docking of the respective substrates, as has been done for P450 2B1.[5]

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