Escherichia coli Expression of Site-Directed Mutants of Cytochrome P450 2B1 from Six Substrate Recognition Sites: Substrate Specificity and Inhibitor Selectivity Studies

You Qun He, You Ai He, and James R. Halpert*

Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, Arizona 85721

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Cytochrome P450 2B1 wild-type and eight site-directed mutations at positions 114, 206, 236, 302, 363, 367, and 478 have been expressed in an *Escherichia coli* system. Solubilized membrane preparations yielded 100–180 nmol of P450/L of culture. The metabolism of a number of substrates including androstenedione, progesterone, (benzyloxy)resorufin, pentoxyresorufin, and benzphetamine was analyzed. The *E. coli*-expressed enzymes displayed the same androstenedione metabolite profiles previously observed with a COS cell expression system. Several of the mutants exhibited an increased rate of progesterone hydroxylation, possibly as the result of an enlarged substrate binding pocket and increased D-ring α-face binding. (Benzyloxy)resorufin and pentoxyresorufin O-dealkylation by the P450 2B1 mutants exhibited activities ranging from 10% to 99% and 3% to 71% of wild-type, respectively. Interestingly, the Val-363 → Leu mutant showed markedly suppressed pentoxyresorufin but unaltered (benzyloxy)resorufin dealkylase activity. Benzphetamine *N*-demethylase activities ranged from 28% to 110% of wild-type. Mechanism-based inactivation of the P450 2B1 mutants showed that susceptibility to inactivation by chloramphenicol and D-*erythro*- and L-*threo*-chloramphenicol was abolished in the Val-367 → Ala mutant. The Val-363 → Leu mutant was refractory to L-*threo*-chloramphenicol. Studies of chloramphenicol covalent binding and metabolism by the Val-367 → Ala mutant showed that its resistance to inactivation is largely attributable to an inability to bioactivate the inhibitor. The expression of P450 2B1 wild-type and mutants in *E. coli* provides an excellent opportunity to study structure/function relationships by site-directed mutagenesis.

Introduction

The cytochrome P450-dependent monooxygenase system plays a key role in the metabolism of a wide variety of xenobiotics and endogenous compounds. Any given P450[1] enzyme usually has the ability to metabolize a number of different substrates, and different P450s often exhibit overlapping specificities (*1*). The question of how the primary sequence encodes the information dictating substrate selectivity is a major area of interest. In recent years, site-directed mutagenesis studies have yielded important information about individual residues responsible for divergent substrate specificity. Many of these studies have utilized as a functional marker the strict regio- and stereoselectivity of steroid hydroxylations catalyzed by certain cytochromes P450. In this manner, a number of laboratories have identified amino acid residues critical for the catalytic specificity of P450 family 2 enzymes. Key residues identified include 117, 209, and 365 in mouse P450 2A subfamily members (*2–4*), residues 114, 206, 290, 302, 363, 367, and 478 in P450 2B enzymes (*5–10*), residues 112–115, 301, 359, and 364 in P450 2C enzymes (*11–17*), and residue 380 in P450 2D1 (*18*). All of these residues fall within or near the six putative substrate recognition sites (SRSs) proposed by Gotoh on the basis of comparative sequence analysis and analogy with bacterial P450 101 (*19*).

Identifying the structural determinants of substrate specificity and inhibitor selectivity of rat P450 2B1[2] has been a major interest of this laboratory in recent years. Up to now, heterologous expression of P450 2B1 mutants in COS cells has provided evidence for an important role for residues 114 (SRS-1), 206 (SRS-2), 302 (SRS-4), 363 and 367 (SRS-5), and 478 (SRS-6) in determining regio- and stereoselectivity of androstenedione (AD) and testosterone metabolism (*6–9*). In contrast, the importance of SRS-3 has remained unsubstantiated (*9*). Appropriate combinations of residues 114, 206, and 478 have been used to convert P450 2B1 from a steroid 16-hydroxylase to a 15α-hydroxylase (*7–9, 20*). However, little is known about the role of individual amino acid residues in the metabolism of substrates other than steroids, although it is clear that the effect of site-directed mutations can be very substrate dependent (*5, 6, 10, 17, 18, 21*). Therefore, a thorough understanding of enzyme specificity will require rigorous biochemical analysis of both wild-

* To whom correspondence and reprint requests should be addressed. Telephone: (520) 626-4358; FAX: (520) 626-2466; E-mail: Halpert@tonic.pharm.arizona.edu.

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[1] Abbreviations: P450, cytochrome P450; SRS, substrate recognition site; AD, androstenedione; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DLPC, dilauryl-L-3-phosphatidylcholine; ALA, δ-aminolevulinic acid.

[2] The P450 2B gene subfamily is composed of cytochromes P450 from different species that have been grouped together on the basis of amino acid sequence identity in accordance with the suggested nomenclature (*30*). This study deals with site-directed mutants of cytochrome P450 2B1. P450 2B1 has the deduced amino acid sequence defined by Fujii-Kuriyama et al. (*31*) except for the first five codons, which are from Suwa et al. (*32*). Genbank accession numbers are J00719 and M11251, respectively.

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type and mutant 2B1 enzymes. Unfortunately, the expression levels previously obtained in COS cells were not adequate for mechanistic studies, and the uncertainty about the exact levels of holoenzyme precludes determination of precise hydroxylation rates. Recently, it has been possible to express P450s 2B in *Escherichia coli* in our laboratory (*22*). In addition to the low cost and relative simplicity of bacterial expression, the amounts of enzyme produced are high enough to permit spectral quantitation and thus determination of catalytic rates of P450 wild-type and mutant enzymes.

In the present investigation, we have expressed cytochrome P450 2B1 wild-type and eight mutant enzymes in an *E. coli* system. CHAPS-solubilized membranes displayed the same androstenedione metabolite profiles found previously with the COS cell system. The metabolism of a number of substrates as well as mechanism-based inactivation by chloramphenicol and two diastereomers have been analyzed. These studies have provided a powerful approach for identifying determinants of substrate specificity and inhibitor selectivity.

Experimental Procedures

Materials. The pKK233-2 *E. coli* expression plasmid was purchased from Pharmacia (Alameda, CA). JM105 and Topp3 cells were purchased from Stratagene (La Jolla, CA). Growth media for *E. coli* were obtained from Difco (Detroit, MI). Restriction endonucleases and DNA modification enzymes were purchased from GIBCO-BRL (Grand Island, NY). Androstenedione, 7-(benzyloxy)resorufin, 7-pentoxyresorufin, resorufin, benzphetamine, formaldehyde, chloramphenicol, NADPH, dimethyl sulfoxide, and CHAPS were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Androstenedione and [^{14}C]-chloramphenicol were purchased from DuPont-New England Nuclear (Boston, MA). TLC plates (silica gel, 250 μM, Si 250 PA (19C)) were obtained from J. T. Baker Inc. (Phillipsburg, NJ). HEPES was purchased from CalBiochem Corp. (La Jolla, CA). *N*-(1,2-Diphenethyl)dichloroacetamide and *N*-(2,2-diphenethyl)dichloroacetamide were synthesized as described (*23*). L-*threo*- and D-*erythro*-chloramphenicol were gifts from Parke Davis Pharmaceuticals (Ann Arbor, MI). All other reagents and supplies not listed were obtained from standard sources.

Construction and Expression of P450 2B1 Wild-Type and Mutant Enzymes in *E. coli*. Selected mutants (I114V, F206L, T302S, V363A, V363L, V367A, and G478S[3]) previously expressed in COS cells from the pBC12BI vector were constructed in the pKK233-2 expression vector as follows. A 1.1-kbp *Pst*I and *Hin*dIII fragment including codons 44−424, or a 374-bp *Bgl*II fragment including codons 360−485 from the wild-type 2B1 cDNA in pKK233-2, was replaced with the corresponding mutated 2B1 cDNA fragment from Bluescript II KS$^-$ (Stratagene) (*7*−*9*). The second codon in the wild-type enzyme had been modified from GAG → GCT to increase expression in *E. coli* (*22*). The identity of the mutated codons in pKK233-2 was confirmed by double-stranded sequencing using a Sequenase 2.0 Kit (United States Biochemical Corp., Cleveland, OH). The U.S.E. Mutagenesis Kit from Pharmacia (Piscataway, NJ) was used for the introduction of a Lys-236 → Asn change in the second-codon modified P450 2B1 cDNA in pKK233-2. The unique restriction enzyme site eliminated in the process of mutagenesis was a *Sal*I site in the vector. The mutated codon was confirmed by double-stranded sequencing using a Sequenase kit. Plasmids harboring the P450 2B1 wild-type and mutant cDNAs were transformed to JM105 or Topp3 cells. *E. coli* were grown and CHAPS-solubilized membranes were prepared as described previously (*22*), except that the P450 concentration in sonicated whole cells was measured daily to identify peak expression levels before membrane preparation.

Steroid Hydroxylation Assays. The assays of steroid hydroxylase activity were performed as described (*10*, *22*) except that 5 pmol of P450 was used in each enzymatic reaction. The final 100-μL reaction mixture contained 10 pmol of rat liver NADPH−cytochrome P450 reductase, 3 μg of DLPC, 10 pmol of rat liver cytochrome b_5, 1 mM NADPH, 2.5 nmol of [^{14}C]AD (50 000 dpm/nmol), or 2.5 nmol of [^{14}C]progesterone (126 984 dpm/nmol) in 50 mM HEPES (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA. 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 dichloromethane/acetone (4:1 v/v) for 15α-OH and 6β-OH androstenedione metabolite separation, and three cycles of chromatography in ethyl acetate/*n*-hexane/acetic acid (16:8:1 v/v) for progesterone metabolites.

(Benzyloxy)resorufin and Pentoxyresorufin O-Dealkylation Assays. The total reaction volume was 500 μL. P450 (20 pmol) was mixed with 40 pmol of rat liver NADPH−cytochrome P450 reductase, 30 μg/mL DLPC, and 20 pmol of rat liver cytochrome b_5. The reconstituted mixture was then incubated for 10 min at room temperature. DLPC (30 μg/mL) in 50 mM HEPES (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA was prepared and added to the reconstituted enzymes. Substrate (10 μM) was added and preincubated for 3 min at 37 °C. The reaction was started by adding NADPH (0.5 mM). The mixture was incubated at 37 °C for 10 min and stopped with 2 mL methanol. Formation of resorufin in the 2.5 mL mixture was monitored by comparison with a standard curve using a Perkin-Elmer Model 3000 spectrofluorimeter. The fluorimeter settings were as follows: excitation slit, 5 nm; excitation wavelength, 550 nm; emission slit, 10 nm; emission wavelength, 585 nm.

Benzphetamine N-Demethylation Assays. P450 (30 pmol) was used in a 300-μL reaction volume. The reaction mixture, containing 60 pmol of rat liver NADPH−cytochrome P450 reductase and 30 μg/mL DLPC, was incubated for 10 min at room temperature. DLPC (30 μg/mL) and rat liver cytochrome b_5 (60 pmol) in 50 mM HEPES (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA were prepared and added to the reconstituted enzymes. Benzphetamine (1 mM) was added and preincubated for 3 min at 37 °C. The reaction was started by adding NADPH (1 mM). The mixture was incubated at 37 °C for 10 min, and then stopped with 300 μL of 10% TCA. The formation of formaldehyde from benzphetamine was measured by the method of Nash (*24*).

Covalent Binding of [^{14}C]Chloramphenicol to 2B1 Wild-Type and the V367A Mutant. P450 (250 pmol) was used in a 1-mL reaction volume. The reaction mixture also contained 500 pmol of rat liver NADPH−cytochrome P450 reductase, 30 μg/mL DLPC, and 500 pmol of rat liver cytochrome b_5. The reconstituted mixture was incubated for 10 min at room temperature. DLPC (30 μg/mL) in 50 mM HEPES (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA was prepared and added to the reconstituted enzymes. [^{14}C]Chloramphenicol (20 000 dpm/nmol) was added to the reconstituted enzymes at a final concentration of 50 μM (*25*). After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 2 mL of ethyl acetate. The tubes were vortexed and then centrifuged (3000 rpm) in a Beckman Model TJ-6 centrifuge. The organic phase was discarded. The extraction procedure was repeated three times. Protein was precipitated by the addition of 3 mL of methanol. The pellet was washed with water and precipitated again with 3 mL of methanol. The supernatants were assayed for ^{14}C with a Beckman LS 2800 liquid scintillation counter. 1 N NaOH (300 μL) was added to the pellet, which was incubated at 60 °C for 1 h. A 10-μL aliquot was taken for protein determination as described (*26*), and 200 μL of the solubilized pellet was neutralized with HCl and taken for scintillation counting.

[3] Eight site-directed mutants are described in the current study. Mutants are indicated as described by Johnson (*33*), using the single-letter code for the amino acid residue replaced, the position in the sequence, and the designation of the new residue, in that order. For example, I114V refers to replacement of Ile at position 114 with Val.

Mechanism-Based Inactivation. P450 2B1 wild-type and mutant enzymes were assayed for residual enzyme activity after incubation with inhibitor in a reconstituted system using [^{14}C]-AD as the substrate. Inactivation studies were performed as described previously (*6*, *7*).

Results

Expression of Rat Liver Cytochrome P450 2B1 Wild-Type and Mutant Enzymes in *E. coli*. To study the molecular basis of substrate specificity and inhibitor selectivity of P450 2B1, expression of large amounts of wild-type and mutant enzymes was required. Therefore, a P450 2B1 wild-type cDNA in pKK233-2, modified at the second codon, was transformed to bacteria. To determine optimal growth conditions for enzyme expression, the effect of different host strains and addition of 80 μg/mL δ-aminolevulinic acid (ALA) was analyzed. The expression levels of P450 were determined by performing a sonicated whole-cell reduced CO/reduced difference spectrum. The results showed that the addition of ALA to the culture causes a substantial increase in expression of P450 in JM105 and Topp3 by 10- and 4-fold, respectively. In addition, the Topp3 strain was found to express more P450 in the absence or presence of ALA than JM105. As a consequence, the expression level varied up to 25-fold among the various conditions, with Topp3 cells grown in the presence of ALA yielding the most P450 (data not shown). In our previous study, CHAPS-solubilized membrane preparations from Topp3 cells grown under such conditions yielded 10–50 nmol of P450 2B1/L of culture (*22*). By monitoring P450 levels in whole cells daily for up to 4 days and harvesting the cells at their peak, expression levels of P450 2B1 of 300–500 nmol/L of culture were routinely obtained in the current study. This resulted in solubilized membrane preparations yielding 100–180 nmol of wild-type P450 2B1/L of culture, or about 30% recovery. Expression levels of all mutants were in the same range as for the wild-type enzyme (data not shown).

Catalytic Activities of P450 2B1 Wild-Type and Mutant Enzymes. Androstenedione has previously been shown to be metabolized efficiently by P450 2B1 wild-type and mutant enzymes in COS cell microsomes (*6–9*). Therefore, androstenedione was used as the initial substrate to verify the enzymatic properties of the *E. coli*-expressed P450s. The P450 2B1 wild-type and seven enzymes with mutations at positions 114, 206, 302, 363, 367, or 478 displayed the same androstenedione metabolite profiles observed previously with COS cell microsomes (Table 1). All mutants except V367A exhibited lower androstenedione hydroxylase activities than the wild-type. One new mutant (K236N) in SRS-3 was also tested based on a report that the corresponding Lys residue in P450 1A1 is important for activity (*27*). This mutant displayed the same metabolite profile and overall rate of androstenedione metabolism as the wild-type enzyme (data not shown) and was not tested further.

Using progesterone, (benzyloxy)resorufin, pentoxyresorufin, and benzphetamine as the substrates, the catalytic activities of P450 2B1 wild-type and seven mutant enzymes were tested (Table 2). In contrast to the results obtained with androstenedione, where hydroxylase activities of the mutants ranged from 9% to 97% of wild-type, four of the mutants, I114V, F206L, T302S, and V363A, exhibited enhanced progesterone hydroxylase activities (148–646% of wild-type). Most interestingly, the size of key residues identified plays a critical role in governing progesterone hydroxylase activity. Compared with the 2B1 wild-type, progesterone hydroxylase activities increase with decreasing size of the side chains at positions 114, 206,[4] 302, and 363, but not position 367, and decrease with increasing size of side chains at position 363 and 478.[4] Using (benzyloxy)resorufin and pentoxyresorufin as the substrates, the reaction rates ranged from 10% to 99% and 3% to 71% of wild-type, respectively. Interestingly, the replacement of Val at position 363 with Leu preferentially suppresses pentoxyresorufin *O*-dealkylase activity, resulting in a (benzyloxy)resorufin:pentoxyresorufin dealkylase ratio 9-fold higher than wild-type. Benzphetamine N-demethylation ranged from 28% to 113% of wild-type, with all mutants except I114V and T302S retaining >50% activity.

Table 1. Metabolism of Androstenedione by Wild-Type and Mutant P450 2B1 Expressed in *E. coli* and COS Cells[a]

	E. coli (nmol/(min·nmol))						ratio			
							16β:16α		15α:16-OH	
	15α	6β	16β	16α	total	%	*E. coli*	COS[e]	*E. coli*	COS[e]
WT[b]	0.4	0.7	38.0	5.1	44.2	100	7.5	8.0	0.01	0.02
I114V	3.0	0.5	9.8	7.6	20.9	47	1.3	1.3	0.2	0.2
F206L	nd[c]	0.4	1.1	1.4	3.9[d]	9	0.8	0.8	nd	0.03
T302S	0.4	0.8	1.9	12.4	15.5	35	0.2	0.2	0.03	0.04
V363A	5.5	1.7	2.8	0.9	10.9	24	3.1	3.4	1.5	1.8
V363L	0.2	0.3	22.2	0.6	23.3	53	37	37	0.01	0.01
V367A	0.4	9.7	31.2	1.6	42.9	97	20	24	0.01	0.01
G478S	1.1	0.6	2.1	2.9	6.7	15	0.7	0.7	0.2	0.2

[a] Values for androstenedione are derived from duplicate incubations performed as described in Experimental Procedures. [b] WT, wild-type. [c] nd, not determined individually. [d] Total activity of F206L includes the sum of 15α-OH AD, 6α-OH AD, and an unknown metabolite. [e] Ratios of 2B1 wild-type and mutants in COS cells were from refs 7–9.

Table 2. Catalytic Activities of P450 2B1 Wild-Type and Mutant Enzymes Expressed in *E. coli*[a]

	progesterone[b]	(benzyloxy)-resorufin	pentoxy-resorufin	benz-phetamine
WT[c]	1.89 (100)[d]	5.73 (100)	4.07 (100)	81.4 (100)
I114V	8.26 (437)	0.65 (11)	1.02 (25)	23.1 (28)
F206L	4.59 (243)	1.81 (32)	1.37 (34)	90.3 (111)
T302S	2.79 (148)	5.4 (94)	2.24 (55)	34.6 (43)
V363A	12.2 (646)	4.42 (77)	2.9 (71)	62.2 (76)
V363L	0.51 (27)	5.67 (99)	0.43 (11)	52.1 (64)
V367A	0.87 (46)	0.97 (17)	0.16 (4)	92.2 (113)
G478S	0.33 (18)	0.56 (10)	0.14 (3)	64.9 (80)

[a] Values for catalytic activities are derived from duplicate incubations performed as described in Experimental Procedures and are expressed as nmol of product/(min·nmol of P450). [b] Total progesterone hydroxylase activity is from 16α-OH + 15α-OH progesterone and 8 unknown metabolites. [c] WT, wild-type. [d] The numbers in parentheses represent the percentage of total activity of wild-type.

Mechanism-Based Inactivation of P450 2B1 Wild-Type and Mutant Enzymes. Mechanism-based inactivators have proven to be valuable probes of functional alterations induced by single amino acid substitutions in P450 2B1 (*6–9*). To explore this further, inactivation of the 2B1 mutants by chloramphenicol (D-*threo*) and its D-*erythro* and L-*threo* diastereomers was also tested. The latter two diastereomers but not the L-*erythro* compound were previously shown to be as effective as chloram-

[4] F206L involves not only a decrease in size but also the replacement of an aromatic with an aliphatic residue, whereas G478S changes a neutral to a polar residue.

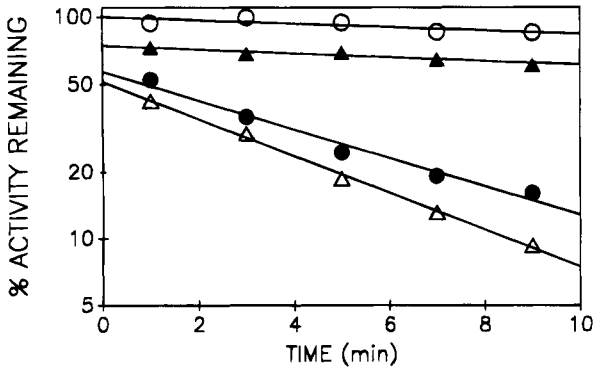

Figure 1. Inactivation of P450 2B1 V363L. (○) methanol control; (△) 500 μM D-*erythro*-chloramphenicol; (●) 50 μM chloramphenicol (D-*threo*); (▲) 500 μM L-*threo*-chloramphenicol. The mutant supplemented with NADPH–cytochrome P450 reductase and cytochrome b_5 was incubated with or without inhibitors for 2 min at 37 °C. Reactions were started by the addition of NADPH. At the times indicated, 80-μL aliquots representing 5 pmol of P450 were removed and added to 20 μL of [^{14}C]androstenedione in buffer. The reactions were allowed to proceed for an additional 5 min and were quenched with 50 μL of tetrahydrofuran. The samples were extracted once with 10 volumes of chloroform, and nine-tenths of the residue from the chloroform extract was redissolved in chloroform and applied to a TLC plate. Inhibitors were added from a methanol stock solution at final concentrations of 50 μM for chloramphenicol and 500 μM for the two diastereomers. The lines shown were generated by linear regression analysis of the natural logarithm of the residual activity as a function of time. For each sample, only the data for the major hydroxylase activity are shown. Rate constants of inactivation are derived from the negative slope of the lines, while the extent of reversible (competitive) inhibition is reflected by a decrease in the extrapolated activity at zero preincubation time compared with the methanol control. The k_i values in min^{-1} were as follows: chloramphenicol (0.16, n = 3), D-*erythro* (0.22, n = 2), L-*threo* (0.03, n = 2), control (0.02, n = 3).

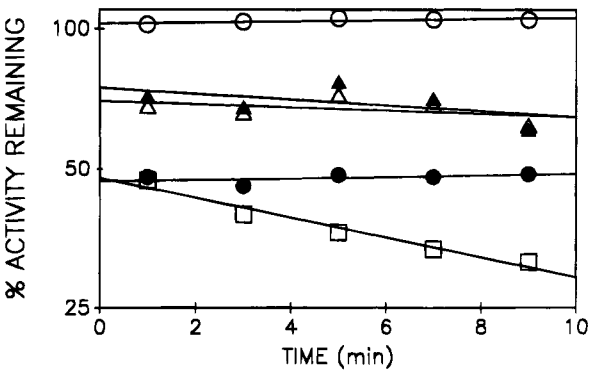

Figure 2. Inactivation of P450 2B1 V367A. (○) methanol control; (△) 500 μM D-*erythro*-chloramphenicol; (●) 500 μM chloramphenicol (D-*threo*); (▲) 500 μM L-*threo*-chloramphenicol; (□) 25 μM *N*-(2,2-diphenethyl)dichloroacetamide. Experiments were performed as described in the legend to Figure 1, using 3 pmol of P450 per time point.

phenicol in inactivating liver microsomal P450 2B1, albeit at a 10-fold higher concentration (*7*). In two independent experiments, wild-type P450 2B1 was inactivated by 50 μM chloramphenicol with a rate constant of 0.16 min^{-1}, the same value previously reported in COS cells (*7*). The rate constant for inactivation of *E. coli*-expressed P450 2B1 by 500 μM D-*erythro*- or L-*threo*-chloramphenicol was 0.13 min^{-1}. In an initial screen of the mutants, I114V, F206L, T302S, V363A, and G478S were inactivated by 50 μM chloramphenicol and 500 μM D-*erythro*-chloramphenicol as rapidly as or more rapidly than the wild-type. In addition, F206L, T302S, and G478S were also inactivated by L-*threo*-chloramphenicol as rapidly as or more rapidly than the wild-type (data not shown). Although V363L was rapidly inactivated by chloramphenicol and its D-*erythro* diastereomer, the mutant was refractory to the L-*threo* compound at a concentration of 500 μM (Figure 1) and concentrations of 1.0 and 2.5 mM (data not shown).

Most strikingly, the substitution of Val with Ala at position 367 renders the enzyme refractory to the two diastereomers and to chloramphenicol itself, even at 500 μM (Figure 2). Previous studies have established that chloramphenicol is metabolized by P450 2B1 to an oxamyl chloride. This intermediate either binds covalently to the enzyme, causing inactivation, or hydrolyzes with water, forming the soluble metabolite chloramphenicol oxamic acid. Inactivation occurs with a stoichiometry of 1.5 nmol of chloramphenicol bound:nmol of P450 inactivated and requires approximately five turnovers of the inhibitor (*25*). To determine the functional basis for the refractory behavior of the V367A mutant, the expressed P450 2B1 wild-type and V367A mutant enzymes were incubated with [^{14}C]chloramphenicol (50 μM) under conditions sufficient to inactivate most of the wild-type enzyme. Covalent binding of chloramphenicol to V367A showed an 11-fold decrease over the wild-type (0.14 nmol of chloramphenicol/nmol of P450 2B1 V367A mutant vs 1.52 nmol of chloramphenicol/nmol of P450 2B1 wild-type). Moreover, soluble metabolites of chloramphenicol were produced by V367A at a 7-fold lower level than wild-type (0.70 vs 4.78 nmol/nmol of P450). Thus, the decreasing size of the side chain at position 367 suppressed chloramphenicol metabolism and covalent binding approximately in parallel.

Two larger chloramphenicol analogs, *N*-(1,2-diphenethyl)dichloroacetamide and *N*-(2,2-diphenethyl)dichloroacetamide, were also tested. As shown in Figure 2, 25 μM *N*-(2,2-diphenethyl)dichloroacetamide caused the same extent of reversible inhibition of the mutant as did 500 μM chloramphenicol. More interestingly, the analog yielded slow but readily detectable time-dependent inhibition of V367A with a rate constant of 0.05 min^{-1}. The corresponding value for 25 μM of the 1,2 analog was 0.07 min^{-1}. The results suggest that the larger size of the analogs may have compensated for a larger binding pocket in the enzyme, thus allowing the inhibitors to be metabolized by and inactivate the V367A mutant.

Discussion

E. coli expression of cytochromes P450 is the most recent heterologous system to be established (ref 22 and references therein). This system provides a facile means for the investigation of P450 structure/function relationships, as it results in large-scale, inexpensive synthesis of functional P450 wild-type and mutant enzymes. A major advantage over the COS cells that we have used extensively to date is that bacterial expression allows precise spectral quantitation of holoenzyme. Thus, the *E. coli* expression system has now enabled a comparison of the rates of androstenedione, progesterone, (benzyloxy)resorufin, pentoxyresorufin, and benzphetamine metabolism among P450 2B1 wild-type and site-directed mutants. Moreover, the availability of adequate amounts of P450 2B1 wild-type and mutants has made it possible to investigate mechanism-based inactivation by chloramphenicol and related compounds as well as chloramphenicol metabolism.

In our previous work using COS cells, the size of the side chains of a number of SRS residues in P450 2B1 was found to play an important role in determining regio- and stereoselectivity of androstenedione and testosterone hydroxylation (*6*–*9*). With both substrates, the β-OH:α-OH ratios decrease with decreasing size of the side chains at positions 114, 206, 302, 363, and 478. The only exception is residue 367, where the β-OH:α-OH ratios increase with decreasing size. The present study with *E. coli*-expressed enzymes confirmed the androstenedione metabolite profiles of these mutants and showed that all mutants except V367A exhibited decreased androstenedione hydroxylase activities. In contrast, mutants I114V, F206L, T302S, and V363A exhibited enhanced progesterone hydroxylation, and V363L, V367A, and G478S decreased progesterone hydroxylation. Most importantly, progesterone hydroxylation increases with decreasing size of the side chains at positions 114, 206, 302, and 363, but not at position 367. The unique results obtained with the latter mutant can be compared with its activity toward androstenedione, where replacement of Val with Ala did not affect total androstenedione β-face (16β + 6β) hydroxylation but decreased 16α-hydroxylase activity 3-fold. P450 2B1 hydroxylates progesterone exclusively on the α-face and at a low rate compared with androstenedione (*5*, *20*), perhaps as the result of steric hindrance of progesterone β-hydroxylation by the 17β-acetyl group. Therefore, the putative enlarged substrate binding pocket in the V367A mutant enzyme may not lead to enhanced overall progesterone hydroxylation if β-hydroxylation is precluded.

The replacement of Val-363 with Leu suppressed 7-pentoxyresorufin O-dealkylation but did not affect 7-(benzyloxy)resorufin O-dealkylation, resulting in a (benzyloxy)resorufin:pentoxyresorufin dealkylation ratio that is approximately 9-fold higher than that of wild-type. Furthermore, V363L was inactivated by 50 μM chloramphenicol and 500 μM D-*erythro*-chloramphenicol as rapidly as the wild-type, whereas the susceptibility to inactivation by L-*threo*-chloramphenicol was abolished. With regard to (benzyloxy)resorufin and pentoxyresorufin metabolism and inactivation by chloramphenicol and diastereomers, the V363L mutant resembles dog P450 2B11,[5] which also has a Leu residue at position 363. In the same vein, it is tempting to speculate that the very low (benzyloxy)resorufin and pentoxyresorufin dealklyase activities of P450 2B5 compared with P450 2B4 (*28*) result in part from the presence of Ala at position 367 in 2B5 as opposed to Val in 2B4.

Benzphetamine is frequently used to determine the activity of cytochrome 2B enzymes in microsomes and purified preparations. However, benzphetamine metabolism proved to be much less sensitive than the other assays to changes at positions 206, 363, 367, and 478, with all these mutants retaining > 60% activity. In fact, it is interesting to speculate that if our original assessment of various 2B mutants had been performed with benzphetamine rather than steroids, some very different conclusions might have been drawn about residues important for substrate specificity. Nonetheless, use of benzphetamine together with the other substrates provides some intriguing findings. For example, there is a 15-fold decrease in the ratio of benzphetamine:progesterone metabolism by I114V compared with wild-type. Overall, by the criteria of altered steroid metabolite profiles (*6*–*10*) or differential changes in total activity with different substrates (*10*, *17*), all the residues examined in the current study remain very likely candidates as actual substrate contact residues.

The most recent efforts in our laboratory have been devoted to elucidation of the role of individual amino acid residues in rat cytochrome P450 2B1 in dictating the susceptibility to chloramphenicol and certain analogs. The studies have established a critical role for amino acid residues 114, 363, 367, and 478 in regulating mechanism-based inactivation (*6*–*9*). In the present investigation, chloramphenicol and two diastereomers were tested for their ability to inactivate seven P450 2B1 mutants. Most interestingly, V363L is refractory to L-*threo*-chloramphenicol and V367A to all three inhibitors. Studies of chloramphenicol covalent binding and metabolism by V367A provided a mechanistic basis for its refractory behavior as largely being attributable to an inability to bioactivate the inhibitor. In contrast, time-dependent inhibition of V367A by the two larger chloramphenicol analogs *N*-(1,2-diphenethyl)dichloroacetamide and *N*-(2,2-diphenethyl)dichloroacetamide was demonstrated. The larger size of the binding pocket accompanying a Val → Ala substitution at position 367 may perhaps be overcome by increasing the size of the inhibitor, thereby increasing the probability that the compound will bind in an orientation that allows metabolism and hence enzyme inactivation. Residues 363 and 367 are hypervariable in P450 2B enzymes (*9*) and may provide a convenient target for design of inhibitors that can distinguish the highly related rat 2B1 from 2B2 or rabbit 2B4 from 2B5.

Very recent computer-generated 3D models of P450 2B1 based on the P450 101 crystal structure are in agreement with the assignment of residues 114, 206, 302, 363, and 478 to the binding pocket for the substrate androstenedione (*29*). The results from progesterone metabolism suggest that this substrate may be docked into the active site of the 2B1 in a manner somewhat different from that of androstenedione or testosterone. Explanations for the differential effects of the mutations observed with the various substrates and inhibitors may come from the P450 2B1 model as has been done for androstenedione.

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