Structural Determinants of Cytochrome P450 2B1 Specificity: Evidence for Five Substrate Recognition Sites†

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ABSTRACT: Twelve site-directed mutants of rat cytochrome P450 2B1 distributed over seven positions and four putative substrate recognition sites (SRS) were constructed and expressed in COS cells. Function was examined using androstenedione and testosterone as substrates. Substitutions at positions 303, 360, and 473 did not markedly affect the regio- or stereoselectivity of androgen metabolism, whereas mutants in positions 206 (SRS-2), 302 (SRS-4), and 363 and 367 (SRS-5) exhibited markedly different steroid metabolite profiles compared with parental P450 2B1. In particular, the Phe-206 → Leu substitution conferred androgen 6α- and testosterone 7α-hydroxylase activities, and the Thr-302 → Ser substitution suppressed androgen 16β-hydroxylation in favor of androstenedione 16α- and testosterone 15α-hydroxylation. Replacement of Val-363 or Val-367 with Ala conferred androgen 15α-hydroxylase and 6β-hydroxylase activities, respectively, and suppressed susceptibility to mechanism-based inactivation by the P450 2B1-selective chloramphenicol analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide. The Val-367 → Ala mutant was also resistant to chloramphenicol itself. The Leu mutant at position 363 exhibited increased specificity for androstenedione and testosterone 16β-hydroxylation, whereas the Leu mutant at position 367 exhibited decreased stereospecificity. Most interestingly, the size of key residues identified plays a critical role in governing steroid hydroxylation from the α-face or β-face and hydroxylation on the D-ring or the B-ring. The findings indicate the importance of residues 206, 302, 363, and 367 in P450 2B1 in determining substrate specificity and regio- or stereoselectivity and, together with previous studies of residues 114 (SRS-1) and 478 (SRS-6), provide experimental evidence for the existence of at least five substrate recognition sites in P450 2B1.

The cytochrome P450 superfamily (Nelson, 1993) is composed of a large group of related enzymes responsible for the oxidation of a wide variety of substrates. Many forms of P450[1] typically exhibit activity toward a broad range of substrates, but in recent years it has become evident that remarkable regio- or stereoselectivity for metabolism of individual substrates is often observed. This is especially true for steroid metabolism by many members of the P450 2 family (Waxman, 1988). The structural basis for such specificity is currently a question of great interest. Recently, Gotoh (1992) proposed the existence of six putative substrate recognition sites (SRS) for the P450 2 family, based on sequence alignments with bacterial P450 101 (Poulos et al., 1987) generated from comparisons of amino acid similarity, hydrophobicity, and predicted secondary structure. To date, all published point mutations that significantly affect substrate specificities and regio- or stereoselectivities of P450 2 forms are located in the SRSs. Key residues identified include 117, 209, and 365 in mouse P450 2A4 and 2A5 (Lindberg & Negishi, 1989; Juvonen et al., 1991; Iwasaki et al., 1993), 114 and 478 in rat P450 2B1 (Aoyama et al., 1989; Kedzie et al., 1991b; He et al., 1992; Halpert & He, 1993), 113 in rabbit P450 2C1, 2C4, and 2C5 (Kronbach & Johnson, 1991; Kronbach et al., 1991), 301 in rabbit 2C2 and 2C14 (Imai & Nakamura, 1989), 364 in rabbit 2C3 (Hsu et al., 1993), 359 in human 2C9 (Kaminsky et al., 1992), and 303 in rabbit 2E1 (Fukuda et al., 1993). All of these residues align with or close to residues in P450 101 shown by X-ray crystallography to constitute part of the substrate binding pocket or access channel (Poulos et al., 1987).

Work in this laboratory has primarily focused on the structural determinants of substrate specificity of P450 2B forms from various species including rat P450 2B1 (Kedzie et al., 1991b; He et al., 1992; Halpert & He, 1993), rabbit P450 2B4 and 2B5 (Kedzie et al., 1991a; Ryan et al., 1993), and dog P450 2B11 (Graves et al., 1990; Kedzie et al., 1991a, 1993). Each of these P450 forms exhibits a unique steroid hydroxylase profile [summarized in Kedzie et al. (1993)]. P450 2B4 and 2B5 differ in only 11 amino acid positions (Gasser et al., 1988) yet exhibit widely different substrate specificities (Kedzie et al. 1991a; Ryan et al., 1993), whereas P450 2B1 and 2B2 differ in 14 positions (Fujii-Kuriyama et al., 1982; Suwa et al., 1985) yet generally exhibit similar specificities (Waxman et al., 1983; Wood et al., 1983) with a few notable exceptions (Christou et al., 1992). All of the substitutions between P450 2B1 and 2B2 lie in the carboxyl-terminal half of the proteins, and six of the residues (303, 360,

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[1] Abbreviations: P450, cytochrome P450; TLC, thin-layer chromatography; IgG, immunoglobulin G; $^-$OH, hydroxy; SDS, sodium dodecyl sulfate; SRS, substrate recognition site.

[2] Nomenclature: 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 (Nelson et al., 1993). This study deals with site-directed mutants of cytochrome P450 2B1 differing at residues 206, 302, 303, 360, 363, 367, and 473. The parental enzyme is referred to as P450 2B1 and has the deduced amino acid sequence defined by Fujii-Kuriyama et al. (1982) except for the first five codons, which are from Suwa et al. (1985) (GenBank accession numbers J00719 and M11251, respectively). Twelve site-directed mutants are described in the current study. Mutants are indicated 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 (Johnson, 1992). For example, F206L refers to replacement of Phe at position 206 with Leu.

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Table 1: Amino Acid Sequences of Cytochromes P450 2B at Six Putative SRS Positions

	SRS-4	SRS-5			SRS-6	
P450	303	360	363	367	473	478
2B1	Ser	Ser	Val	Val	Lys	Gly
2B2	Gly[a]	Ala	Ala	Leu	Met	Ala
2B4	Thr	Gly	Ile	Val	Arg	Gly
2B5	Thr	Gly	Val	Ala	Arg	Gly
2B11	Thr	Gly	Leu	Val	Gln	Gly

[a] Amino acid substitutions made in this investigation are underlined.

363, 367, 473, and 478) lie within SRS-4, SRS-5, and SRS-6 (Table 1). Pairwise comparisons of the other functionally distinct 2B enymes also reveal some interesting similarities and differences at these same sites (e.g., 2B4/2B5 or 2B5/2B11 at positions 363 and 367). Interestingly, a Gly → Ala substitution in position 478 (which converts the 2B1 residue to the 2B2 residue) confers unique functional properties on P450 2B1 (Kedzie et al., 1991b; He et al., 1992). To understand better the structural basis for functional similarities and differences between the rat 2B enzymes, nine P450 2B1 mutants at an additional five putative SRS positions were constructed (Table 1). In addition, mutations were made at residues 206 (SRS-2) and 302 (SRS-4), which are conserved among the cytochromes P450 2B but align to residues known to be of critical functional significance in other P450 2 enzymes. All mutants were expressed in COS cells and examined for androstenedione and testosterone hydroxylase activities. This has led to the identification of amino acid residues 206, 302, 363, and 367 in P450 2B1 as important determinants of substrate specificity and regio- or stereoselectivity.

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 *Escherichia 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). The Gene Clean kit was obtained from Bio 101 (La Jolla, CA). Androstenedione, 16α-OH androstenedione, chloramphenicol, NADPH, chloroquine, dimethyl sulfoxide, 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). [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 was purchased 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). *N*-(2-*p*-Nitrophenethyl)chlorofluoroacetamide was synthesized as described (Stevens & Halpert, 1988; Halpert et al., 1990). COS cells were obtained from Dr. John Regan (University of Arizona, Tucson, AZ). All other reagents and supplies not listed were obtained from standard sources.

Site-Directed Mutagenesis. The introduction of specific base changes in the P450 2B1 cDNA was accomplished using a Muta-Gene Phagemid in vitro mutagenesis kit from Bio-Rad (Richmond, CA). Twelve oligonucleotide primers were synthesized, and their sequences are shown in Table 2. All mutated codons were confirmed by double-stranded sequencing using a Sequenase 2.0 kit from U.S. Biochemical Corp. (Cleveland, OH). Following mutagenesis, inserts were excised from Bluescript II KS$^-$ (Stratagene, San Diego, CA) with *Sal*I and *Sma*I and subcloned into pBC12BI (Cullen, 1987) for expression in COS cells.

Table 2: Nucleotide Sequences of P450 2B1 Mutagenic Primers

primer	sequence[a]
F206L	GG ACC TTg TCC CTC CTA AGT TC
T302S	C ACT GAG tCC AGC AGC ACC ACA CTC C
T302V	C ACT GAG gtC AGC AGC ACC ACA CTC C
S303T	C ACT GAG ACC AcC AGC ACC ACA CTC C
S303G	C ACT GAG ACC gGC AGC ACC ACA CTC C
S360A	CAG AGG TTT gCA GAT CTT GTC CC
S360G	CAG AGG TTT ggA GAT CTT GTC CC
V363L	CA GAT CTT cTC CCT ATT GGA G
V363A	CA GAT CTT GcC CCT ATT GAA G
V367L	C CCT ATT GGA cTA CCA CAC AGA G
V367A	C CCT ATT GGA GcA CCA CAC AGA G
K473M	C CTC ACG CCC AtG GAG AGT GGC

[a] The lower-case letters denote the changed nucleotides.

Expression in COS Cells and Microsome Preparation. COS cells were grown and transfected as described previously (Kedzie et al., 1991b). COS cell microsomes were prepared 72 h posttransfection as described previously (Kedzie et al., 1991b), including a washing step as described (He et al., 1992).

Steroid Hydroxylase Assays. The assays of androstenedione hydroxylase activity (Graves et al., 1987; Kedzie et al., 1991b) and testosterone hydroxylase activity (Ciaccio & Halpert, 1989) were performed as described. 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 or testosterone metabolite separation. Quantification of 15α-OH and 6α-OH androstenedione was achieved as described (Kedzie et al., 1991a; Halpert & He, 1993). The identity of 7α-OH testosterone was confirmed by oxidation to the androstenedione derivative with β-hydroxysteroid dehydrogenase at pH 9.

Immunochemical Methods. SDS–Polyacrylamide gels (7.5%) were run as described (Laemmli, 1970). Transfer of proteins to nitrocellulose and immunodetection were performed as described (Kedzie et al., 1991a,b).

RESULTS

Heterologous Expression in COS Cells. Twelve site-directed mutants of rat cytochrome P450 2B1 were constructed. Nine of these involved single amino acid substitutions at five putative SRS positions (303, 360, 363, 367, and 473) where P450 2B1 and 2B2 differ. In each case, the 2B1 residue was mutated to the corresponding residue in 2B2 to yield the mutants S303G, S360A, V363A, V367L, and K473M. One additional mutant was also made at each of these sites except position 473. The mutants S303T and S360G were chosen to yield the consensus 2B residue among the rabbit 2B4 and 2B5 and dog 2B11 sequences (Table 1), and mutants V363L and V367A were selected to give the 2B11 and 2B5 residues, respectively. In addition to the nine mutants at positions that exhibit considerable variability among the 2B enzymes, mutants were made at two conserved residues known to be of functional significance in other family 2 enzymes. The mutant F206L mimics the Phe-209 → Leu substitution in P450 2A5, which is known to confer steroid 15α-hydroxylase activity (Lindberg & Negishi, 1989), and the mutants T302S and

T302V explore the role of the side chain on the residue that corresponds to Thr-252, which forms part of the oxygen binding pocket in P450 101 (Poulos et al., 1987). All mutated cDNAs were subcloned into the expression vector pBC12BI and expressed in COS cells. COS cell microsomes were analyzed by immunoblotting for expression levels and assayed for androstenedione and testosterone hydroxylation. All mutants were expressed at similar levels to each other and to wild-type P450 2B1 except for S360G, which was approximately 2-fold lower (data not shown).

Steroid Hydroxylase Activities of 303, 360, 363, 367, and 473 Mutants. P450 2B1 hydroxylates androstenedione primarily at the 16β-position and to a lesser extent the 16α-position, whereas with testosterone, equal amounts of the 16β-OH and 16α-OH products are formed (Waxman et al., 1983; Wood et al., 1983; He et al., 1992). None of the five mutants at positions 303, 360, or 473 exhibited significant alterations in the stereospecificity of androstenedione or testosterone 16-hydroxylation, nor were any novel metabolites formed (data not shown). In contrast, substitutions at residues 363 and 367 caused major changes in the androstenedione (Figure 1 (top); Table 3) and testosterone (Figure 1 (bottom); Table 4) metabolite profiles. With both substrates, replacement of Val-363 by Leu preferentially suppresses 16α-hydroxylase activities, resulting in androstenedione and testosterone 16β-OH:16α-OH ratios that are 4- and 14-fold higher than parental P450 2B1, respectively. The Val-363 → Ala substitution suppresses androgen 16β- and 16α-hydroxylase activities and enhances 15α-hydroxylase activities. As a consequence, the androstenedione and testosterone 15α-OH:16-OH ratios increase approximately 100- and 300-fold, respectively. In contrast to the results observed with Val-363, the substitution Val-367 → Leu suppresses androgen 16β-hydroxylase activities more than 16α-hydroxylase activities, such that the 16β-OH:16α-OH ratios decrease. Interestingly, substitution of Val-367 with Ala confers considerable androstenedione 6β- and testosterone 6β-hydroxylase activities, and the mutant still catalyzes hydroxylation at the 16β- and 16α-positions. An intriguing observation is the *decrease* in the androgen β-OH:α-OH ratios with decreasing size of residue 363 but the *increase* in the β-OH:α-OH ratios with decreasing size of residue 367 (Table 5).

Steroid Hydroxylase Activities of 206 and 302 Mutants. P450 2B1, 2B2, 2B4, 2B5, and 2B11 hydroxylate androstenedione and testosterone almost exclusively on the D-ring (Kedzie et al., 1993). Replacement of Phe-206, which is conserved in all five enzymes, with Leu in P450 2B1 markedly suppresses androgen 16β-hydroxylase activities and confers androstenedione and testosterone 6α-hydroxylase and testosterone 7α-hydroxylase activities (Figure 1; Tables 3 and 4). Accordingly, B-ring hydroxylations account for at least 29% of product formation from androstenedione or testosterone catalyzed by F206L. Thus, the presence of a conserved Phe at this position in P450 2B enzymes may help direct androstenedione and testosterone hydroxylation to the steroid D-ring. Marked changes in steroid hydroxylase activities were also observed upon substitution of another conserved residue, Thr-302, which was replaced with Ser or Val. The T302S mutant produces 16α-OH androstenedione (80%) and 15α-OH testosterone (58%) as major metabolites, and 16β-hydroxylase activities are markedly suppressed with both substrates (Figure 1; Tables 3 and 4). As a consequence, the androstenedione 16β-OH:16α-OH ratio is approximately 40-fold lower in the T302S mutant compared with parental P450 2B1, and the testosterone 15α-OH:16-OH ratio is 140-fold higher than the wild type. Substitution of Thr-302 with Val significantly suppresses androstenedione and testosterone hydroxylase activities (5% and 8% of total activities of parental P450 2B1, respectively). The Val mutant exhibits an altered testosterone hydroxylase profile (16β-OH:16α-OH = 0.2) compared with the wild type (16β-OH:16α-OH = 1) but little difference with androstenedione. The data suggest that both the CH_3^- and OH^- groups on the Thr-302 side chain contribute to the rate and specificity of androstenedione and testosterone hydroxylation by P450 2B1.

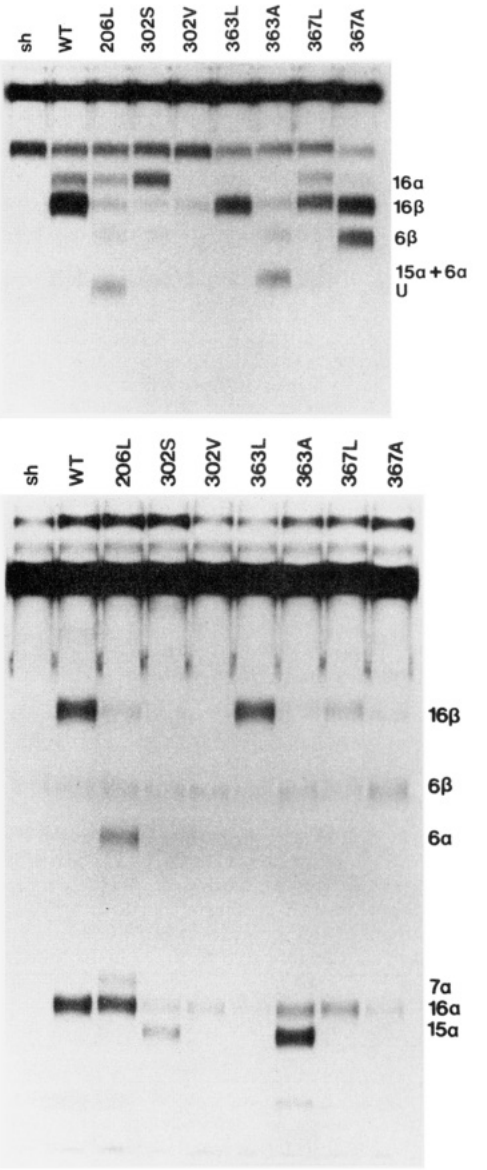

FIGURE 1: Autoradiogram of androstenedione (top) or testosterone (bottom) metabolites produced by microsomes from transfected COS cells. Microsomal protein (100 μg) was incubated with 25 μM [^{14}C]-androstenedione or 200 μM [^{14}C]testosterone for 30 min at 37 °C in 100 μL of buffer containing 20 pmol of NADPH–cytochrome 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. Samples are designated by the position in the sequence and the one-letter code for the substituted amino acid residue. The 15α + 6α/U bands were recovered and reduced to the corresponding testosterone metabolites for better separation. The data are shown in Tables 3 and 4. WT, wild type; sh, sham-transfected.

Mechanism-Based Inactivation of P450 2B1 Mutants. As a complement to steroid substrates, mechanism-based inactivators have proven valuable probes of functional alterations induced by single amino acid substitutions (Kedzie et al.,

Table 3: Androstenedione Metabolism by Microsomes from Transfected COS Cells[a]

enzyme	androstenedione hydroxylase activity[b] [pmol min^{-1} mg^{-1} (%)]					ratio	
	15α-OH	16β-OH	16α-OH	6β-OH	total activity	16β-OH:16α-OH	15α-OH:16-OH
WT[c]	4.6 (2)	244 (87)	30.5 (11)	2.5 (1)	281.6	8.0	0.02
F206L[d]	1.0 (2)	15.9 (25)	20.3 (32)	5.2 (8)	62.5	0.8	0.03
T302S	2.3 (4)	8.2 (12)	52.3 (80)	3.0 (4)	65.8	0.2	0.04
T302V	0.8 (5)	12.4 (81)	2.1 (14)	nd[e]	15.3	5.9	0.06
V363L	0.9 (1)	80.5 (96)	2.2 (3)	nd	83.6	37	0.01
V363A	24.8 (57)	10.5 (24)	3.1 (7)	5.2 (12)	43.6	3.4	1.8
V367L	3.6 (5)	52.1 (71)	17.3 (24)	nd	73.0	3.0	0.05
V367A	1.3 (1)	115 (65)	4.8 (3)	54.8 (31)	175.9	24	0.01

[a] COS cell microsomes (100 μg) were incubated as described in the legend to Figure 1 and Experimental Procedures. Values for the individual hydroxylase activities represent the means from duplicate incubations after correction for appropriate blanks from incubations with control COS microsomes. [b] The numbers in parentheses represent the percentage of total androstenedione hydroxylase activity. [c] The means ± SD for the percentages of 15α-OH, 16β-OH, 16α-OH, and 6β-OH metabolites from six experiments with wild-type P450 2B1 are 1.6 ± 0.2, 86.2 ± 1.0, 11.2 ± 0.9, and 1.0 ± 0.1, respectively. The values for the 16β-OH:16α-OH and 15α-OH:16-OH ratios are 7.7 ± 0.7 and 0.02 ± 0.00, respectively. [d] 6α-Hydroxylase and unknown hydroxylase activities were detected, 16.8 (27%) and 3.3 (5%) pmol min^{-1} mg^{-1}, respectively. [e] No 6β-hydroxylation detectable.

Table 4: Testosterone Metabolism by Microsomes from Transfected COS Cells[a]

enzyme	testosterone hydroxylase activity[b] [pmol min^{-1} mg^{-1} (%)]				ratio	
	15α-OH	16β-OH	16α-OH	total activity	16β-OH:16α-OH	15α-OH:16-OH
WT[c]	2.0 (1)	76.1 (49)	77.6 (50)	155.7	1.0	0.01
F206L[d]	3.6 (3)	12.2 (11)	64.9 (57)	114.7	0.2	0.05
T302S	17.8 (58)	3.8 (12)	9.1 (30)	30.7	0.4	1.4
T302V	0.9 (7)	1.8 (14)	10.0 (79)	12.7	0.2	0.08
V363L	0.2 (<1)	59.3 (93)	4.2 (7)	63.7	14	<0.01
V363A	101 (74)	7.6 (6)	27.0 (20)	135.6	0.3	2.9
V367L	1.7 (4)	13.7 (35)	23.6 (60)	39	0.6	0.05
V367A[e]	0.3 (2)	3.9 (21)	6.8 (36)	18.7	0.6	0.03

[a] COS microsomes (100 μg) were incubated as described in the legend to Figure 1 and Experimental Procedures. Values for the individual hydroxylase activities represent the means from duplicate incubations after correction for appropriate blanks from incubations with control COS microsomes.[b] The numbers in parentheses represent the percentage of total testosterone hydroxylase activity. [c] The means ± SD for the percentage of 15α-OH, 16β-OH, and 16α-OH metabolites from eight experiments with wild-type P450 2B1 are 0.8 ± 0.5, 51.2 ± 1.6, and 48.0 ± 1.5, respectively. The values for the 16β-OH:16α-OH and 15α-OH:16-OH ratios are 1.1 ± 0.1, and 0.01 ± 0.01, respectively. [d] 6α- and 7α-hydroxylase activities were detected, 21.9 (19%) and 12.1 (10%) pmol min^{-1} mg^{-1}, respectively. [e] 6β-Hydroxylase activity was detected, 7.7 (41%) pmol min^{-1} mg^{-1}.

Table 5: Androgen Metabolite Ratios of P450 2B1 Mutants

position	substitution	ratio		
		testosterone β-OH:α-OH	androstenedione β-OH:α-OH	androstenedione D-ring OH: B-ring OH
114	I → V	0.04	0.9	50
	I → A	0.01	0.2	4.3
206	F → L	0.1	0.6	1.7
302	T → S	0.1	0.2	21
363	V → L	13.5	26	nd[a]
	V → A	0.06	0.6	7.4
367	V → L	0.5	2.5	nd
	V → A	1.6	28	2.2
478	G → L	2.1	7.0	nd
	G → V	1.1	2.9	nd
	G → A	0.4	0.9	nd
WT[b]		1.0	6.8	100

[a] No B-ring hydroxylation detectable. [b] WT represents parental P450 2B1.

Table 6: Rate Constants for Inactivation of P450 2B1 Mutants[a]

P450	k_i (min^{-1})	
	chloramphenicol	*N*-(2-*p*-nitrophenethyl)-chlorofluoroacetamide
2B1	0.16[b]	0.13[b]
V363L	0.10, 0.14	0.10, 0.12
V363A	0.10, 0.11	0.01, 0.03,[c] 0.04[c]
V367L	0.05, 0.05	0.07, 0.08
V367A	0.00, 0.00[c]	0.02, 0.00[c]

[a] Experiments were carried out as described (He et al., 1992) using 50 μM inhibitor unless otherwise indicated. Rate constants of inactivation were derived by linear regression analysis of the natural logarithm of the residual activity as a function of time. Values represent the results from independent experiments. Control values (no inhibitor) were all ≤0.02 min^{-1}. [b] From He et al. (1992). [c] Inhibitor concentration = 250 μM.

1991b; He et al., 1992; Halpert & He, 1993). Because of the interesting effect of the size of residues 363 and 367 on steroid hydroxylase profiles, inactivation of the four mutants at these positions was also examined. As shown in Table 6, V363L was inactivated by 50 μM chloramphenicol and 50 μM of the P450 2B1-selective analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide almost as rapidly as parental P450 2B1. The mutant V363A was also readily inactivated by chloramphenicol but was refractory to the analog. The susceptibility to inactivation by both inhibitors was diminished by replacement of Val-367 with Leu and was abolished by replacement of Val-367 with Ala. Even a 5-fold higher concentration of either inhibitor failed to inactivate the V367A mutant. The results extend our previous studies of residues 114 and 478, which showed that inactivation of P450 2B1 by *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide is much more sensitive to single amino acid substitutions than inactivation by chloramphenicol. This correlates well with the observation that the analog is selective for P450 2B1 and 2B2, whereas chloramphenicol inactivates a number of other family 2 and family 3 enzymes (Stevens & Halpert, 1988; Halpert et al., 1990).

DISCUSSION

Heterologous expression of P450 2B1 mutants in COS cells has provided evidence for an important role for residues 206, 302, 363, and 367 in determining substrate specificity as well

as regio- and stereoselectivity. Residue 206 in P450 2B1 corresponds to residue 209 in P450 2A5 or 2A4, and the substitution Phe-209 → Leu confers the steroid 15α-hydroxylase activity of 2A4 on 2A5 (Lindberg & Negishi, 1989). Gotoh (1992) maps this residue to SRS-2. Recent sequence alignments and modeling studies assign residue 209 in P450 2A5 to either the end of helix F or the F–G loop (Iwasaki et al., 1993). The helix and loop constitute part of the substrate pocket and/or substrate-access channel in P450 101 (Poulos et al., 1987). According to the 2A5 model, the 11β-OH of various steroids appears to interact with residue 209 located in the substrate binding pocket. In the current study, replacement of Phe-206 in P450 2B1 with Leu was found to confer androstenedione and testosterone 6α-hydroxylase and testosterone 7α-hydroxylase activities (B-ring), activities not detectable in parental P450 2B1, while maintaining 16β-, 16α-, and 15α-hydroxylase activities (D-ring). Alteration of steroid metabolite profiles by this single amino acid substitution indicates that residue 206 plays a pivotal role in determining the catalytic specificity of P450 2B1 and is consistent with the model proposed by Iwasaki et al. (1993).

Thr-252 in P450 101 is located in helix I close to the 6th ligand position and plays a critical role in the proper formation of the oxygen binding pocket (Poulos et al., 1987). Several studies suggest that the corresponding conserved Thr in mammalian P450s plays a similar role and is also involved in determining substrate specificity and regioselectivity (Imai & Nakamura, 1989; Hanoika et al., 1992; Fukuda et al., 1993). For example, a Thr-303 → Ser substitution in P450 2A1 causes a 2-fold increase in the V_{max} for testosterone 7α-hydroxylation, most likely as the result of more efficient coupling of NADPH and oxygen utilization to product formation (Hanoika et al., 1992). With P450 2E1 (Fukuda et al., 1993) but not 2C2 (Imai & Nakamura, 1989), replacement of the conserved Thr by Ser affects the regioselectivity of lauric acid hydroxylation, whereas with caprate as a substrate, the same regioisomers are produced by the mutated P450 2E1 as by the wild type. In the present study, it was found that substitution of Thr-302 (SRS-4) with Ser in P450 2B1 changes the stereoselectivity of androstenedione and testosterone 16-hydroxylation as well as the regioselectivity of testosterone hydroxylation. Replacement of the conserved Thr with Val in P450 2C2 and 2E1 does not change the fatty acid hydroxylase profiles, but the activities are markedly decreased. Likewise, a decrease in testosterone and progesterone 16α-hydroxylase activities is observed upon substitution of Thr-301 with Val in P450 2C14 (Imai & Nakamura, 1989). In P450 2B1, substitution of Thr-302 with Val suppresses androstenedione 16β- and 16α-hydroxylase activities in parallel. With testosterone, 16β-hydroxylase activity is suppressed to a greater extent than 16α-hydroxylase activity, such that stereoselectivity is altered 5-fold. The findings with the Ser and Val mutants suggest that Thr-302 in P450 2B1 plays a critical role in controlling steroid metabolism. This could occur by influencing substrate binding *per se* or by facilitating cleavage of the dioxygen bond and preventing hydrogen peroxide release (Poulos et al., 1987; Hanoika et al., 1992). In particular, the enhanced testosterone 15α-hydroxylase activity of the Ser mutant is suggestive of altered binding orientation, whereas the changed stereoselectivity of androstenedione 16-hydroxylation with the Ser mutant and of testosterone 16-hydroxylation with the Val mutant suggests preferential suppression of the efficiency of hydroxylation of the β- as opposed to α-face of the substrate. Overall, the sensitivity of parental 2B1 and the T302S and T302V mutants to the difference between a 17-keto group (androstenedione) and 17β-OH group (testosterone) suggests close proximity between residue 302 in P450 2B1 and the C-17 position.

In the present study, six mutants at positions 360, 363, and 367 within SRS-5 were also investigated. According to the sequence alignments between cytochrome P450 family 2 enzymes and P450 101, Val-363 in P450 2B1 corresponds to one of the proposed substrate contact residues in P450 101, Val-295 (Poulos et al., 1987; Gotoh, 1992). Substitution of Val-363 or Val-367 in P450 2B1 with Leu or Ala has a profound effect on androstenedione and testosterone hydroxylase profiles. The Ala mutants in particular exhibit novel hydroxylase activities (V363A = 15α-OH; V367A = 6β-OH) and are refractory to inactivation by the P450 2B1-selective analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide. Studies of other family 2 enzymes have also reported single amino acid substitutions in this region that alter catalytic activities. A Leu-365 → Met substitution in P450 2A4 (corresponding to position 362 in P450 2B1) confers the coumarin hydroxylase activity of P450 2A5 (Lindberg & Negishi, 1989), and a Ser-364 → Thr substitution in P450 2C3 (corresponding to position 366 in 2B1) yields progesterone 6β-hydroxylase activity (Hsu et al., 1993). In P450 2C9, an Ile-359 → Leu substitution alters the regio- and stereoselectivity of warfarin hydroxylation from 7-hydroxylation of (*S*)-warfarin to 4′-hydroxylation of (*R*)-warfarin (Kaminsky et al., 1992). Interestingly, replacement of the corresponding Ser-360 in P450 2B1 to Gly or Ala does not alter the regio- or stereoselectivity of androstenedione or testosterone metabolism. A fourth SRS-5 mutation, Ile-380 → Phe in P450 2D1 (corresponding to position 370 in 2B1), restores the selective loss of bufuralol metabolism to a mutant form, 2D1v (Matsunaga et al., 1990). In general, the results indicate excellent agreement among the studies with the different family 2 enzymes and suggest that P450 101 is an appropriate model for these mammalian enzymes in the region surrounding Val-295, as suggested (Poulos, 1991). In addition, the profound effects of single amino acid substitutions in SRS-5 of P450 2B1 on mechanism-based inactivation suggest that minor structural differences between P450 2 enzymes in this region may provide a means for design of specific inhibitors.

An intriguing question is how P450 2B1 and 2B2 can exhibit similar regio- and stereoselectivity of androstenedione and testosterone metabolism, given the marked effects of the 2B1 → 2B2 substitutions at positions 363 and 367 (*vide supra*) as well as 478 (Kedzie et al., 1991; He et al., 1992). To test whether some combination of 2B1 → 2B2 substitutions at these residues might restore the high androstenedione 16β-OH:16α-OH ratio characteristic of P450 2B1 and 2B2 (Waxman, 1983; Wood et al., 1983), two double mutants (V363A/G478A and V367L/G478A) and one triple mutant (V363A/V367L/G478A) were constructed and expressed in COS cells. However, all three mutants exhibited significantly altered androstenedione metabolite profiles compared with P450 2B1 (data not shown). This finding suggests that the functional role of these three residues may be modified by (1) other SRS residues, such as 303, 360, or 473, single substitution of which does not affect activity, or (2) non-SRS residues, which were not evaluated in the current study.

In our previous work, the size of the side chains of residues 114 and 478, which are located within SRS-1 and SRS-6, respectively, was found to play an important role in determining regio- and stereoselectivity of androgen hydroxylation (Kedzie et al., 1991b; He et al., 1992; Halpert & He, 1993). In the present study, the size of residues 206, 302, 363, and 367 was

also established as a key determinant of specificity. With both androstenedione and testosterone, the β-OH:α-OH ratios decrease with decreasing size of the side chains at positions 114, 206, 302, 363, and 478. The only exceptions are residue 367, where the β-OH:α-OH ratios increase with decreasing size, and the Gly → Ala mutant at residue 478 (Table 5). Decreasing the size of the side chains of residues 114, 206, 363, and 367 also enhances hydroxylation of the B-ring of androstenedione (Table 5). These substitutions are likely to enlarge the size of the substrate binding pocket and to allow for alternate binding orientations. Altered binding orientation is also likely to account for the refractory behavior of several of the mutants to mechanism-based inactivation by the P450 2B1-selective analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide. Overall, our studies on P450 2B1 have now provided experimental evidence for the existence of five substrate recognition sites in P450 2B1, although it cannot be excluded that the functional consequences of some of the amino acid substitutions reflect indirect effects on substrate contact residues that are adjacent in the three-dimensional but not primary structure. Key residues identified through mutagenesis of P450 2B1 include 114 (SRS-1), 206 (SRS-2), 302 (SRS-4), 363 and 367 (SRS-5), and 478 (SRS-6). Only the proposed SRS-3 (Gotoh, 1992) remains unsubstantiated by site-directed mutagenesis.[3] The present investigation has also provided a strong basis for further studies to redesign P450 2B1 specificity.

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[3] Because of the high sequence conservation among cytochromes P450 2B in the putative SRS-3 (residues 234–241) and the lack of mutagenesis data from other family 2 enzymes, no mutants were made in this region. However, neither the results of this investigation nor the title of the article are meant to imply that there are only five SRS in P450 2B1, not six as proposed by Gotoh (1992).

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