Role of Residue 478 as a Determinant of the Substrate Specificity of Cytochrome P450 2B1†

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ABSTRACT: Two allelic variants and eight site-directed mutants of cytochrome P450 2B1 differing at residue 478 have been expressed in COS cells and assayed for androstenedione hydroxylase activities. The 478Gly and 478Ala variants and five mutants (Ser, Thr, Val, Ile, and Leu) exhibited 16β-OH:16α-OH ratios ranging from 0.7 to 9.3, whereas the Pro, Glu, and Arg mutants were expressed but inactive. The seven samples active toward androstenedione also exhibited testosterone 16β-OH:16α-OH ratios ranging from 0.4 to 2.3. With both steroids, the Gly variant had the highest 16β-hydroxylase activity, and the 16β-OH:16α-OH ratio increased with the size of aliphatic size chains (Ala, Val, and Ile/Leu). The highest ratio of androgen 15α:16-hydroxylation was observed with the Ser mutant. On the basis of previous work indicating decreased susceptibility of the 478Ala variant in liver microsomal and reconstituted systems to inactivation by chloramphenicol analogs, methodology was refined for monitoring enzyme inactivation in COS cell microsomes. The Gly and Ala variants were inactivated by chloramphenicol with similar rate constants, whereas the Ser and Val mutants were inactivated more slowly, and the Leu mutant was refractory. Only the Gly variant was inactivated by the chloramphenicol analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide. Thus, the side chain of residue 478 appears to be a major determinant of enzyme inactivation as well as of androgen hydroxylation. Overall, this study demonstrates the importance of the amino acid at position 478 in dictating the substrate specificity of P450 2B1 and provides firm experimental evidence for a model in which this residue comprises part of a substrate recognition site.

The molecular basis for the diverse substrate specificities of individual members of the cytochrome P450 superfamily is currently a question of great interest (Johnson, 1992). Although no three-dimensional structure has been reported for a mammalian cytochrome P450, models based on sequence alignments with bacterial P450[1] 101 (Poulos et al., 1987) have begun to reveal putative substrate recognition sites (Laughton et al., 1990; Zvelebil et al., 1991; Gotoh, 1992). In addition, recent studies with site-directed mutants and hybrid enzymes have yielded important information regarding individual residues responsible for substrate specificity (Lindberg & Negishi, 1989; Aoyama et al., 1989; Imai & Nakamura, 1989; Matsunaga et al., 1990; Kronbach & Johnson, 1991; Kedzie et al., 1991b; Johnson, 1992; Sligar et al., 1992). Especially useful in this regard has been the comparison of allelic variants, proteins encoded by the same genetic locus in different strains or individuals of the same species and containing one or more amino acid substitutions. Allelic variants with different catalytic properties provide an excellent means for pinpointing individual amino acid residues of potential functional importance. By this approach, residues 58 and 114 in P450 2B1[2] (Aoyama et al., 1989), residue 380 in P450 2D1 (Matsunaga et al., 1990), and residue 364 in P450 2C3 (Johnson, 1992) have recently been identified as important determinants of substrate specificity of cytochromes P450 belonging to family 2 (Nebert et al., 1991). Furthermore, site-directed mutagenesis of P450 2A4 and 2A5, which are products of different genes but exhibit only 11 amino acid substitutions, has revealed the crucial importance of residues 117, 209, and 365 in determining substrate specificity (Lindberg & Negishi, 1989; Juvonen et al., 1991).

Our work has focused on an allelic variant of P450 2B1 isolated from rats of the inbred WM strain (Kedzie et al., 1991b). This variant exhibits a 10-fold lower androstenedione 16β:16α-hydroxylation ratio compared with other P450 2B1 preparations. In addition, P450 2B1-WM is refractory to mechanism-based inactivation by the chloramphenicol analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide, a selective inactivator of other 2B1 variants. A cDNA encoding P450 2B1-WM was cloned and sequenced, revealing a single amino acid substitution (Gly 478 → Ala) (Kedzie et al., 1991b) compared with the published sequence (Fujii-Kuriyama et al., 1982). Expression of P450 2B1 and P450 2B1-WM in COS cells confirmed that residue 478 is responsible for the unusual stereoselectivity of androstenedione 16-hydroxylation catalyzed by P450 2B1-WM. The molecular basis for this remained obscure, although we speculated that the tendency

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[1] Abbreviations: WM, Wistar Munich; LEW, Lewis; TLC, thin-layer chromatography; P450, cytochrome P450; IgG, immunoglobulin G; -OH, hydroxy; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCN, pregnenolone-16α-carbonitrile.

[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 (Nebert et al., 1991). This study deals with allelic variants and site-directed mutants of cytochrome P450 2B1 differing at residue 478. The major variant is referred to as P450 2B1 or the Gly variant 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). The variant expressed in inbred WM rats is identical in deduced amino acid sequence to P450 2B1 except for an Ala instead of a Gly residue at position 478 (Kedzie et al., 1991b) and is referred to as P450 2B1-WM or the Ala variant. Eight site-directed mutants are described and are referred to as the Ser mutant, Thr mutant, Val mutant, etc.

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of Ala to stabilize and of Gly to destabilize α-helices (Lyu et al., 1990; O'Neil & DeGrado, 1990) might be involved. Furthermore, until recently it was not possible to probe definitively the role of this residue in dictating the susceptibility of P450 2B1 to mechanism-based inactivation. In the current study, we substituted residue 478 in P450 2B1 with eight amino acids of varying size, charge, hydrophobicity, and helix-stabilizing tendencies and examined the androstenedione and testosterone hydroxylase activities of the resultant mutants. Methodology for assessing mechanism-based inactivation in COS cell microsomes has also been refined. These studies establish that the nature of the side chain of residue 478 in cytochrome P450 2B1 is a critical determinant of androgen hydroxylation and of susceptibility to mechanism-based inactivation by chloramphenicol and analogs.

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, Dulbecco's modified Eagle's medium, penicillin–streptomycin, and trypsin–EDTA were purchased from Gibco-BRL (Grand Island, NY). 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. N. Kirk, University of London (London, England). [4-^{14}C]-Androst-4-ene-3,17-dione and [4-^{14}C]testosterone were purchased from DuPont–New England Nuclear (Boston, MA). TLC plates [silica gel, 250 μM, Si 250 PA (19C)] were purchased 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 purchased from Pharmacia Fine Chemicals (Piscataway, NJ). *N*-(2-*p*-Nitrophenethyl)chloroacetamide, *N*-(2-*p*-nitrophenethyl)-chlorofluoroacetamide, *N*-(2,2-diphenethyl)chlorofluoroacetamide, *N*-(2-phenethyl)dichloroacetamide, and *N*-(2,2-diphenethyl)dichloroacetamide were synthesized as described (Stevens et al., 1988; Halpert et al., 1990). L-*threo*-, D-*erythro*-, and L-*erythro*-chloramphenicol were gifts from Parke Davis Pharmaceuticals (Ann Arbor, MI). COS cells were obtained from Dr. John Regan (University of Arizona, Tucson, AZ). Rats were obtained from Harlan/Sprague-Dawley (Indianapolis, IN). All other reagents and supplies not listed were obtained from standard sources.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using the Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad, Richmond, CA). Oligonucleotide primers used were GTGGCATTtCAAAAATACCTCC (Ala478Ser), GTGGCATTaCAAAAATACCTCC (Ala478Thr), GTGGCATTGtAAAAATACCTCC (Ala478Val), GTGGCATTcTAAAAATACCTCC (Val478Leu), GTGGCATTaTAAAAATACCTCC (Val478Ile), GTGGCATTcCAAAAATACCTCC (Ala478Pro), GTGGCATTGaAAAAATACCTCC (Ala478Glu), and GTGGCATTaGAAAAATACCTCC (Gly478Arg). Lower-case letters denote the mutant nucleotides. The sequences of the mutated P450 2B1 cDNAs were confirmed by double-stranded sequencing using a Sequenase kit (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.

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) except that microsomes were collected by centrifugation at 150000*g* for 30 min and washed by resuspension in HEPES buffer (50 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, and 0.1 mM EDTA) followed by recentrifugation. Final suspension was in 10 mM Tris acetate, pH 7.4, 20% glycerol, and 1 mM EDTA.

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. The identification of 15α-OH androstenedione was confirmed by enzymatic conversion to 15α-OH testosterone using β-hydroxysteroid dehydrogenase as described previously (Kedzie et al., 1991a). The 15α-OH testosterone was identified by two cycles of thin-layer chromatography in dichloromethane/acetone (4:1 v/v) or sequential chromatography in dichloromethane/acetone (4:1 v/v) and chloroform/ethyl acetate/100% ethanol (4:1:0.7 v/v/v) as described (Waxman, 1991).

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).

Treatment of Animals and Preparation of Microsomes. Male LEW and WM rats (200–250 g) were pretreated for 5 days with 0.1% sodium phenobarbital in drinking water and were killed by cervical dislocation. Livers were removed immediately and used to prepare microsomes as described previously (Halpert et al., 1983). Protein was determined as described (Lowry et al., 1951).

RESULTS

Androstenedione Hydroxylase Activities. P450 2B1, P450 2B1-WM (Kedzie et al., 1991b), and eight site-directed mutants at position 478 were expressed in COS cells. Initial in situ assays of androstenedione hydroxylation (Kedzie et al., 1991b) indicated that the Ser, Thr, Val, Ile, and Leu mutants were expressed in enzymatically active form, whereas no activity was observed with the Pro, Glu, or Arg mutants (data not shown). To achieve a more quantitative measure of the expression levels and activities of the various mutants, COS cell microsomes were prepared. As evidenced by immunoblotting, all the mutants were expressed (Figure 1), and no obvious differences in electrophoretic mobility were noted. This is in agreement with previous findings that the heterologously expressed Ala and Gly variants comigrate, despite differences in the electrophoretic mobilities of P450 2B1 variants in liver microsomes (Kedzie et al., 1991b). Consistent with the in situ assays, no androstenedione hydroxylase activity was observed with the microsomes

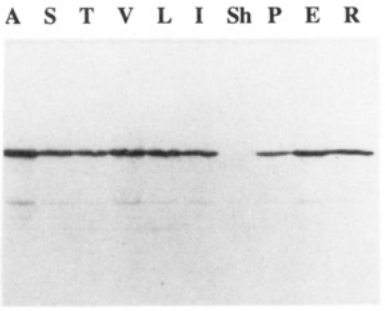

FIGURE 1: Immunological detection of heterologously produced P450 2B1 mutants in COS cell microsomes. Microsomal proteins (20 μg) were separated on an SDS–polyacrylamide gel, followed by electrotransfer to nitrocellulose. Proteins were detected with rabbit anti-P450 2B1 IgG (primary antibody), goat anti-rabbit IgG conjugated to alkaline phosphatase (secondary antibody), and a color reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Lane A, P450 2B1-WM; lane S, Ser mutant; lane T, Thr mutant; lane V, Val mutant; lane L, Leu mutant; lane I, Ile mutant; lane Sh, sham transfected; lane P, Pro mutant; lane E, Glu mutant; lane R, Arg mutant.

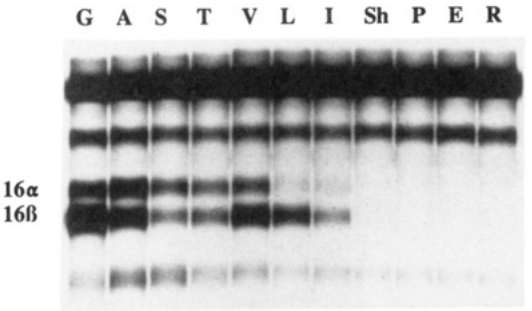

FIGURE 2: Autoradiogram of androstenedione metabolites produced by microsomes from transfected COS cells. Transfected COS cell microsomal protein (200 μg) was incubated with 25 μM [^{14}C]-androstenedione for 30 min at 37 °C in 100 μL of buffer containing 20 pmol of NADPH–cytochrome P450 reductase and 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. Lane G, P450 2B1; lane A, P450 2B1-WM; lane S, Ser mutant; lane T, Thr mutant; lane V, Val mutant; lane L, Leu mutant; lane I, Ile mutant; lane Sh, sham transfected; lane P, Pro mutant; lane E, Glu mutant; lane R, Arg mutant.

expressing the Pro, Glu, or Arg mutants[3] (Figure 2), despite the fact that levels of immunoreactive protein were comparable with those of the active mutants. Because expression levels were found to vary approximately 2-fold among the different mutants or among different preparations of COS microsomes expressing the same variant or mutant, absolute activities normalized per miligram of protein (Table I) can be interpreted only semiquantitatively as a measure of the intrinsic activity of each expressed protein. However, metabolite profiles for each mutant were consistent regardless of the expression level. While caution must be used in interpreting rates of the various hydroxylations, metabolite profiles provide a very reliable and sensitive index of changes in enzyme function induced by alterations of the side chain of residue 478.

With the above caveat, a number of interesting trends were observed in overall androstenedione hydroxylase activities and

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

amino acid residue[b]	androstenedione hydroxylase activity (pmol min^{-1} mg^{-1})			ratio	
	15α-OH	16β-OH	16α-OH	16β-OH: 16α-OH	15α-OH: 16-OH
Gly	2.5	117	15.8	7.4	0.02
Ala	2.4	14.0	14.8	0.9	0.08
Ser	1.8	3.8	5.4	0.7	0.20
Thr	0.9	5.0	5.4	0.9	0.09
Val	0.3	14.6	4.8	3.0	0.02
Ile	0.2	2.2	0.3	7.3	0.08
Leu	0.3	8.4	0.9	9.3	0.03

[a] Results represent the mean of two analyses, performed in duplicate. [b] Activities of the Pro, Glu, and Arg mutants are <0.1.

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

amino acid residue[b]	testosterone hydroxylase activity (pmol min^{-1} mg^{-1})			ratio	
	15α-OH	16β-OH	16α-OH	16β-OH: 16α-OH	15α-OH: 16-OH
Gly	0.8	35.2	42.0	0.8	0.01
Ala	0.8	5.4	14.2	0.4	0.04
Ser	1.1	1.7	3.2	0.5	0.22
Thr	0.3	1.8	3.8	0.5	0.05
Val	0.1	2.8	2.4	1.2	0.02
Ile	0.0	1.4	0.6	2.3	*c*
Leu	0.0	3.8	1.8	2.1	*c*

[a] Results represent the mean of one analysis, performed in duplicate. [b] Activities of the Pro, Glu, and Arg mutants were not determined. [c] Not calculated.

metabolite profiles of the Ser, Thr, Val, Ile, and Leu mutants when compared with the Gly and Ala variants (Kedzie et al., 1991b). As shown in Figure 2 and Table I, substitution of Gly 478 by Ala in P450 2B1 markedly decreased androstenedione 16β-hydroxylase activity with little or no effect on 16α- or 15α-hydroxylase activities. In addition, the Ser and Thr mutants retained less than 5% of the 16β-hydroxylase activity but approximately $^1/_3$ of the 16α-hydroxylase and $^2/_3$ or $^1/_3$, respectively, of the 15α-hydroxylase activity of the Gly variant. These results strongly suggest that the Gly residue plays a critical role in determining specific 16β-hydroxylation in P450 2B1. Another interesting observation is the relatively high ratio of 15α:16-hydroxylation catalyzed by the Ser mutant. The most obvious trend observed with the four mutants with aliphatic side chains (Ala, Val, Ile, and Leu) is an increase in the 16β-OH:16α-OH ratio with increasing size.[4]

Testosterone Hydroxylase Activities. Because of the intriguing alterations in androstenedione hydroxylation induced by substitution of residue 478 in P450 2B1, the metabolism of testosterone by various P450 2B1 mutants was examined (Table II). Replacement of the keto group in the C-17 position of androstenedione with the hydroxyl group in testosterone decreases the stereoselectivity of 16-hydroxylation by reconstituted P450 2B1 approximately 10-fold (Waxman et al., 1983; Wood et al., 1983; Kedzie et al., 1991b). Consistent with these results, the testosterone 16β-OH:16α-OH ratio of the expressed Gly variant is approximately 10-fold lower than the androstenedione metabolite ratio. In general, hydroxylase activities and metabolite profiles were less sensitive to alterations of residue 478 with testosterone

[3] Microsomes from sham-transfected COS cells form two products from androstenedione. One product migrates between the parent compound and 16α-OH androstenedione in both TLC systems used and has the same mobility as testosterone. The second product migrates more slowly than 16β-OH androstenedione and has the same mobility in dichloromethane/acetone (4:1 v/v) as 6β-OH androstenedione. The Pro, Glu, and Arg mutants of P450 2B1 produced no androstenedione metabolites in excess of those attributable to the COS cells alone.

[4] The relative sizes of the amino acid residues substituted at position 478 are Gly < Ala < Ser < Thr < Val < Leu/Ile. Some ambiguity exists as to the relative sizes of Leu and Ile (Moody & Wilkinson, 1990; Lehrman, 1990).

as a substrate than with androstenedione, although the major trends were similar. Thus, (1) testosterone 16β-hydroxylase activity is greatly diminished upon substitution of Gly 478, (2) the 16β-OH:16α-OH ratio is the highest with the larger aliphatic residues, and (3) the Ser mutant exhibits the highest ratio of 15α-OH:16-OH formation.

Inactivation of P450 2B1 by Chloramphenicol in COS Cells. Previous studies in liver microsomes and reconstituted systems indicated that the function of P450 2B1 from WM rats is unique not only with regard to the stereoselectivity of androgen hydroxylation but also in terms of susceptibility to mechanism-based inactivation by chloramphenicol analogs. These results suggested that a single amino acid substitution might be sufficient to dictate the inactivation of a cytochrome P450. Pilot experiments were devoted to the development of a system for evaluating the inactivation of P450 2B1 in COS cells. A 1-h in situ preincubation of COS cells with 250 μM chloramphenicol followed by extensive washing decreased the androstenedione 16β-hydroxylase activity of expressed P450 2B1 by 52% compared with cells washed immediately after addition of the inhibitor (data not shown). Although a time-dependent inhibition of P450 2B1 by chloramphenicol was demonstrated, the in situ response was slow compared with rate constants of inactivation determined in microsomes or reconstituted systems (Kedzie et al., 1991b; Stevens & Halpert, 1988) and was not deemed sufficiently sensitive to detect the potentially subtle differences in mechanism-based inactivation of the P450 2B1 mutants.

Preliminary studies of inactivation of P450 2B1 by chloramphenicol in COS microsomes prepared as described previously (Kedzie et al., 1991b) yielded a rate constant of inactivation (k_i) of 0.07 min^{-1} (Figure 3), a value 4-fold lower than observed in liver microsomes. We reasoned that this might reflect poor electron transfer, despite the addition of exogenous rat liver NADPH–cytochrome P450 reductase and cytochrome b_5. Therefore, cholate solubilization was tried in order to achieve better incorporation of these auxiliary proteins into the microsomal membrane. Accordingly, COS microsomes were mixed with the reductase and cytochrome b_5 and stirred on ice for 30 min in buffer containing or lacking 0.6% sodium cholate, and the samples were dialyzed to remove the cholate. In the course of these experiments it was determined that, regardless of cholate treatment, the dialyzed microsomes exhibited twice their original androstenedione 16β-hydroxylase activity and were efficiently inactivated by chloramphenicol with a k_i of 0.16 min^{-1}. Other experiments revealed that a washing step with HEPES buffer during preparation of COS cell microsomes as described under Experimental Procedures was as effective as dialysis in unmasking the susceptibility of expressed P450 2B1 to mechanism-based inactivators. Therefore COS cell microsomes were routinely washed prior to all subsequent inactivation experiments. Additional experiments were designed to confirm that the rate constant of inactivation of P450 2B1 by chloramphenicol is independent of the expression level, such that comparisons of inactivation constants among 2B1 mutants would be valid. Western blots of representative preparations of COS P450 2B1 microsomes revealed a direct correlation between immunoreactivity and androstenedione 16β-hydroxylase activity (data not shown). Although expression levels normally varied only 2-fold (vide supra), one sample of COS 2B1 microsomes was obtained that exhibited an 8-fold lower expression level than that normally observed. However, even in this extreme case, the rate constant for inactivation of P450 2B1 by chloramphenicol was unaltered (Figure 3).

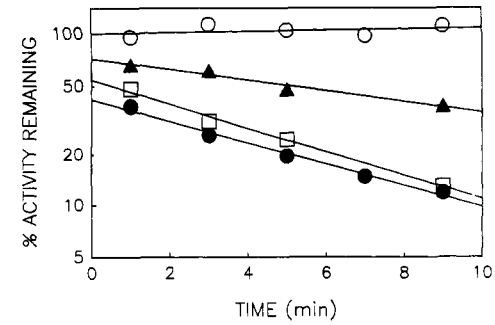

FIGURE 3: Effect of washing or dialysis of COS microsomes on inactivation by 50 μM chloramphenicol of P450 2B1-mediated androstenedione 16β-hydroxylase activity. ○, methanol control, dialyzed high-activity microsomes; ▲, unwashed high-activity microsomes; ●, dialyzed high-activity microsomes; □, washed low-activity microsomes. Unwashed microsomes were prepared from sonicated COS cells by differential centrifugation, and the microsomal pellet was resuspended in 10 mM Tris acetate, pH 7.4, 20% glycerol, and 1 mM EDTA. Washed microsomes were prepared by resuspension of the initial microsomal pellet in 50 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, and 0.1 mM EDTA, followed by centrifugation and resuspension in the Tris buffer. Dialyzed microsomes were prepared from a mixture of unwashed microsomes, rat liver NADPH–cytochrome P450 reductase, and cytochrome b_5, which was dialyzed for 4 h against four changes of incubation buffer. Unwashed or washed microsomes, reductase, and cytochrome b_5 were incubated together for 10 min at room temperature. Samples in incubation buffer with and without chloramphenicol were warmed for 2 min at 37 °C. Reactions were started by the addition of NADPH. At the times indicated, 80-μL aliquots 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. One-third (50 μL) of the total volume was applied to a TLC plate. The amounts of the various components of the incubation after addition to androstenedione were 25 μM androstenedione, 150–500 μg of microsomal protein, 20 pmol of reductase, and 10 pmol of cytochrome b_5. Chloramphenicol was added from a methanol stock solution, and the concentration indicated refers to the preincubation period prior to addition of the substrate. The lines shown were generated by linear regression analysis of the natural logarithm of the residual activity as a function of time. In this type of assay, unmetabolized inhibitor is present during the assays of substrate metabolism. Rate constants of inactivation are derived from the negative slope of the lines, while the extent of competitive inhibition is reflected by a decrease in the extrapolated activity at zero preincubation time (y intercept) compared with the controls. The androstenedione 16β-hydroxylase activities of the various preparations in picomoles per minute per milligram of protein were as follows: high-activity unwashed (106), high-activity washed (237), and low-activity washed (32).

Selective Inactivation of P450 2B1 Variants and Mutants. Microsomes prepared from COS cells expressing P450 2B1-WM were assayed for residual androstenedione 16β-hydroxylase activity after preincubation with chloramphenicol (Figure 4A). Consistent with previous results in reconstituted systems, the expressed Ala variant of P450 2B1 was inactivated by chloramphenicol with a similar rate constant as the expressed Gly variant. Inactivation of the Ser, Val, and Leu mutants by chloramphenicol was also assessed (Figure 4A), and the susceptibility to chloramphenicol inactivation was found to decrease as the size of the residue at position 478 increased, such that the Leu mutant is refractory to the compound. The Ser mutant was inactivated by chloramphenicol with a rate constant intermediate between those of the Ala and Val mutants, suggesting that the side-chain hydroxyl group of the Ser residue does not influence the inactivation process. Only the Gly variant was inactivated by *N*-(2-*p*-nitrophenethyl)-chlorofluoroacetamide, although reversible inhibition was observed with the Ala variant and Ser mutant as evidenced by the decrease in activity at zero preincubation time (Figure 4B). The results clearly demonstrate that a single amino acid

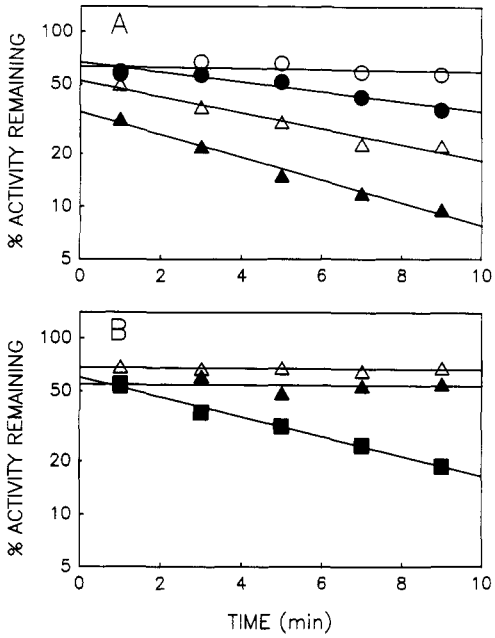

FIGURE 4: Effect of preincubation with inhibitors on the major androstenedione hydroxylase activity of P450 2B1 variants and mutants. (A) Chloramphenicol. ○, Leu mutant; ●, Val mutant; △, Ser mutant; ▲, Ala variant. (B) *N*-(2-*p*-Nitrophenethyl)chlorofluoroacetamide. △, Ser mutant; ▲, Ala variant; ■, Gly variant. Experiments were carried out as described in the legend to Figure 3 using 50 μM inhibitor except for the Leu mutant, where 250 μM chloramphenicol was used. Incubates from reactions with the Leu and Ser samples were extracted once with 1 mL of chloroform following quenching with tetrahydrofuran. Nine-tenths of the residue from the chloroform extract was redissolved in chloroform and applied to a TLC plate. The k_i values derived from the graphs and expressed in reciprocal minutes are as follows: (A) Leu (0.01), Val (0.06), Ser (0.11), and Ala (0.15); (B) Ser (0.00), Ala (0.00), and Gly (0.13).

substitution at position 478 can dictate the susceptibility of P450 2B1 to mechanism-based inactivation.

Inactivation of P450 2B1 Variants in Rat Liver Microsomes. The lack of inactivation of reconstituted P450 2B1-WM by *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide has been attributed to inability of the enzyme to catalyze the oxidative dehalogenation reaction required for reactive metabolite formation (Kedzie et al., 1991b). This could reflect an inadequate oxidation potential for hydroxylation of the chlorofluoroacetamido group or improper orientation of the inhibitor at the active site. To explore this further, additional analogs and three diastereomers of chloramphenicol were tested for their ability to inactivate P450 2B1-WM. For simplicity, experiments were performed with liver microsomes from phenobarbital-treated WM rats instead of from transfected COS cells. For purposes of comparison, liver microsomes from phenobarbital-treated LEW rats were also included. These express the P450 2B1 variant examined in most of our previous studies of the structural requirements for P450 inactivation by chloramphenicol analogs (Stevens & Halpert, 1988; Halpert et al., 1990; Kedzie et al., 1991b).

The ability to discriminate between the P450 2B1 variants is not restricted to chlorofluoroacetamides, as evidenced by the effects of compounds B, C, and F (Table III). In addition, with a second phenyl group on the ethyl side chain, a chlorofluoroacetamide is able to inactivate P450 2B1-WM (compound E), and a dichloroacetamide (compound C) that does not inactivate P450 2B1-WM is rendered capable of inactivating the enzyme (compound D). Overall, the results with the chloramphenicol analogs suggest that the nature of the substituents on the ethyl side chain is a major determinant

Table III: Rate Constants for Inactivation of P450 2B1 Variants by Analogs and Stereoisomers of Chloramphenicol[a]

	compound	concn (μM)	k_i (min^{-1}) LEW	k_i (min^{-1}) WM
	control (methanol)		0.00[b]	0.00[c]
	chloramphenicol	50	0.30	0.30
A	*N*-(2-*p*-nitrophenethyl)-chlorofluoroacetamide	250	0.16	0.02
B	*N*-(2-*p*-nitrophenethyl)-chloroacetamide	250	0.16	0.02
C	*N*-(2-phenethyl)dichloro-acetamide	25	0.14	0.00
D	*N*-(2,2-diphenethyl)-dichloroacetamide	5	0.13	0.11
E	*N*-(2,2-diphenethyl)-chlorofluoroacetamide	50	0.08	0.08
F	L-*threo*-chloramphenicol	500	0.26	0.08
G	D-*erythro*-chloramphenicol	500	0.19	0.17
H	L-*erythro*-chloramphenicol	500	0.04	0.00

[a] Microsomes prepared from the livers of phenobarbital-treated male LEW or WM rats were incubated with and without inhibitors in the presence of NADPH. The incubations and assays of residual androstenedione 16β-hydroxylase activity were carried out essentially as described in the legend to Figure 3, except that incubations lacked added reductase and cytochrome b_5, the amount of microsomal protein was 25 μg (LEW) or 50 μg (WM), and the androstenedione hydroxylation reactions were terminated after 1.5 min. Rate constants for inactivation (k_i) were calculated as described for Figure 3. Each value represents the average of two or three determinations. The 100% activities in nanomoles of metabolite per minute per milligram of microsomal protein were 24 (LEW) and 4 (WM). [b] $n = 4$. [c] $n = 3$.

of inactivation of P450 2B1-WM. The inability of L-*erythro*-chloramphenicol to inactivate either variant is also consistent with the interpretation that interaction of P450 2B1 with substitutents on the ethyl side chain of the inhibitor is an important determinant of enzyme inactivation.

DISCUSSION

Heterologous expression of P450 2B1 mutants in COS cells has been used in conjunction with steroid substrates and mechanism-based inactivators to probe the role of residue 478 in governing substrate specificity. Focus on this residue was prompted by a recent study from this laboratory, which indicated that a Gly → Ala substitution at position 478 is responsible for the unique functional properties of an allelic variant of P450 2B1 from WM rats (Kedzie et al., 1991b). The use of steroid metabolite profiles and rate constants for inactivation, both of which are independent of expression levels, circumvented problems inherent in the use of COS cells, such as variable transfection efficiencies and limitations in the determination of the precise amount of active enzyme (Kronbach & Johnson, 1991). With these reporter probes, the functional consequences of replacement of Gly 478 in P450 2B1 with amino acid side chains of varying size, charge, hydrophobicity, and helix-stabilizing tendencies were explored. The results confirm that Gly 478 confers unique functional properties on P450 2B1, including high androstenedione and testosterone 16β-hydroxylase activities and susceptibility to inactivation by certain chloramphenicol analogs. The conformational freedom of Gly and its small size are likely to be the major factors involved.

With regard to other substitutions at position 478 in P450 2B1, a number of parallels can be drawn with the results of a recent investigation in which Phe 209 in P450 2A5 was replaced with eight other amino acids, including Leu, Val, Ala, and Ser (Juvonen et al., 1991): (1) In both cases, the size and hydrophobicity of the side chain are critical deter-

minants of enzyme function. With increased size of aliphatic amino acids at residue 478 in P450 2B1, the androstenedione 16β-OH:16α-OH ratio increases and susceptibility to mechanism-based inactivation by chloramphenicol decreases. In P450 2A5, the K_m values for coumarin 7-hydroxylation decrease and the V_{max} values increase with increasing size of residue 209. (2) The Ser mutants of P450 2B1 and 2A5 exhibit hydroxylase activities and profiles that cannot be explained solely on the basis of size, suggesting some specific influence of the side-chain hydroxyl group. (3) Acidic residues, Asp in the case of P450 2A5 and Glu in the case of P450 2B1, abolish activity in a way that cannot be attributed to size alone, suggesting a specific influence of the negatively charged side chain. (4) Helix-stabilizing tendencies appear not to make a major contribution to the observed functional differences among the various mutants of P450 2B1 or 2A5. Thus, Leu and Ala are very similar with regard to helix stabilization (O'Neil & DeGrado, 1991), yet the functional properties of the Leu and Ala mutants at residue 478 in P450 2B1 or at residue 209 in P450 2A5 are very different. In summary, site-directed mutagenesis of residue 209 in P450 2A5 and of residue 478 in 2B1 reveals a number of common properties of these residues, suggesting that each participates in a substrate recognition site in its respective enzyme, as recently proposed (Gotoh, 1992).

The precise function of residue 478 in P450 2B1 is not known; however, positions 471–478 map to a central region of β-sheet 5 in P450 101 that includes residues 395–396 (Poulos et al., 1987; Gotoh, 1992). Although these residues do not contact the substrate (Poulos et al., 1987), they are within 10 Å of the bound camphor molecule (Laughton et al., 1990) and may constitute part of a substrate access channel (Poulos et al., 1987). The corresponding residues in the mammalian cytochromes P450 could also form part of a substrate access channel and/or could contribute directly to substrate binding (Johnson, 1992). The latter interpretation is supported by the results of the present investigation, which suggest that residue 478 in P450 2B1 plays a major role in dictating the binding of androgens, chloramphenicol, and chloramphenicol analogs. In particular, the distinct stereo- and regioselectivity of androgen D-ring hydroxylation catalyzed by the Ser and Thr mutants is suggestive of altered binding orientation, perhaps due to hydrogen bonding between the side-chain hydroxyl group and the 3-keto group on the A-ring of the substrate.

The final key finding of the present investigation is the demonstration that a single amino acid substitution in P450 2B1 can dictate the susceptibility of the enzyme to mechanism-based inactivation by chloramphenicol and analogs. In recent years, several laboratories including our own have promoted the use of mechanism-based inactivators as highly selective probes of P450 function (CaJacob et al., 1988; Muerhoff et al., 1989; Halpert et al., 1990), with the rationale that the requirement for catalysis adds an extra degree of isoform selectivity compared with inhibitors that rely solely on binding. With the very recent realization that a single amino acid substitution can drastically alter the catalytic activities of many cytochromes P450 (reviewed in Johnson, 1992), there is an increased need for chemical agents that can discriminate between functionally different cytochromes P450 that are nearly identical in structure. Mechanism-based inactivators appear to be uniquely suited for this purpose. In fact, several apparently anomalous findings in the literature may be readily explained on the basis of the ability of mechanism-based inactivators to discriminate between highly related cytochromes P450. For example, the fact that only 50% of the activity of a purified preparation of P450 4A1 is susceptible to 10-undecynoic acid (CaJacob et al., 1988) could reflect the presence of an allelic variant or another member of the 4A subfamily that copurifies with the 4A1. Likewise, our previous finding that chloramphenicol differentially affects marker activities of P450 3A1 in liver microsomes from PCN-treated female rats (Graves et al., 1987) may reflect the presence of allelic variants in the outbred animals. The use of heterologous expression systems to monitor enzyme inactivation as described for P450 2B1 should prove the ideal means of clarifying these issues. In addition, the ability to manipulate enzyme as well as inhibitor structure should lend a new level of sophistication to the rational design of specific mechanism-based inactivators of individual cytochrome P450 isoforms and allelic variants.

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