CYP4 Isozyme Specificity and the Relationship between ω-Hydroxylation and Terminal Desaturation of Valproic Acid†

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ABSTRACT: The cytochrome P450-dependent terminal desaturation of valproic acid (VPA) is of both toxicological and mechanistic interest because the product, 4-ene-VPA, is a more potent hepatotoxin than the parent compound and its generation represents a rather novel metabolic reaction for the cytochrome P450 system. In the present study, lung microsomes from rabbits were identified as a rich source of VPA desaturase activity. Monospecific polyclonal antibodies directed against CYP4B1 (anti-4B) inhibited 82% of 4-ene-VPA formation, whereas monospecific polyclonal antibodies directed against CYP2B4 (anti-2B) inhibited only 15% of 4-ene-VPA formation. Anti-4B also inhibited 95% of the 5-hydroxy-VPA formation, but only 42% of 4-hydroxy-VPA formation. These data suggest that CYP4B1 accounts for more than 80% of the 4-ene- and 5-hydroxy-VPA metabolites generated by rabbit lung microsomes. CYP4B1 expressed in HepG2 cells metabolized VPA with a turnover number of 35 min^{-1} and formed the 5-hydroxy-, 4-hydroxy-, and 4-ene-VPA metabolites in a ratio of 110:2:1, respectively. In contrast, the lauric acid ω-hydroxylases, CYP4A1 and CYP4A3, did not give rise to detectable levels of any of these VPA metabolites. Therefore, these studies demonstrate a new functional role for CYP4B1 in the terminal desaturation and ω-hydroxylation of this short, branched-chain fatty acid. Intramolecular deuterium isotope effects on the formation of 4-ene-VPA, 4-hydroxy-VPA, and 5-hydroxy-VPA by cDNA-expressed CYP4B1 and by purified, reconstituted CYP2B1 indicate that a carbon-centered free radical at C-4 serves as a common intermediate for P450-dependent formation of both 4-ene-VPA and 4-hydroxy-VPA, regardless of whether the enzyme source functions preferentially as an ω-hydroxylase or an ω-1 hydroxylase of VPA. The partition between oxygen rebound to, and desaturation proceeding from, the C-4 VPA radical was 2:1 for CYP4B1 and 37:1 for the VPA 4-hydroxylase CYP2B1. Therefore, active site constraints which promote facile ω-hydroxylation bias the partition ratio toward desaturation and significantly enhance the rate of P450-dependent 4-ene-VPA formation.

Cytochrome P450 (CYP450)[1] catalyzes a wide variety of well-recognized oxidative reactions, including aliphatic and aromatic hydroxylation, *N*- and *O*-dealkylation, and *S*-oxygenation. In recent years, awareness of the functional versatility of this enzyme has been heightened by reports that ester hydrolysis (Guengerich, 1987), *N*-oxygenation of tertiary amines (Seto & Guengerich, 1993), and desaturation of aliphatic and alicyclic substrates (Nagata et al., 1986; Rettie et al., 1987, 1988; Korzekwa et al., 1990; Vyas et al., 1990; Guengerich & Kim, 1991; Yost & Skiles, 1992; Kassahun & Baillie, 1993) also are components of the enzyme's metabolic repertoire.

In addition to the expected ω, ω-1, and ω-2 alcohols generated from VPA by NADPH-supplemented rat liver microsomes (Prickett et al., 1984), we have identified two olefinic metabolites, 3-ene- and 4-ene-VPA (Rettie et al., 1987) (Figure 1). The reaction leading to the latter product, which has been shown to be catalyzed by cytochrome P450 (Rettie et al., 1987, 1988), is of toxicological interest because terminal olefins are widely recognized as precursors to reactive species, capable of inactivating a wide range of enzymes, often by mechanism-based processes (Ortiz de Montellano, 1988). Indeed, the terminal olefins methylenecyclopropylacetic acid and 4-pentenoic acid have featured prominently in this regard as experimental hepatotoxins due to their ability to act as inhibitors of fatty acid β-oxidation (Bressler et al., 1969; Billington et al., 1978; Schulz, 1983). 4-ene-VPA, the 2-*n*-propyl derivative of pentenoic acid, has also attracted considerable interest, because it was proposed that this terminal olefin might be implicated in the etiology of an idiosyncratic, sometimes fatal, hepatotoxicity caused by administration of VPA (Zimmerman & Ishak, 1982), characteristic features of which are mitochondrial dysfunction and severe impairment of fatty acid β-oxidation enzymes.

Although the specific biochemical mechanisms by which VPA causes liver dysfunction are not understood, several

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[1] Abbreviations: CYP450, cytochrome P450; VPA, valproic acid, 2-*n*-propylpentanoic acid; 4-ene-VPA, 2-*n*-propyl-4-pentenoic acid; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; GC/FID, gas chromatography–flame ionization detection; GC/MS, gas chromatography–mass spectrometry; TMS, trimethylsilyl.

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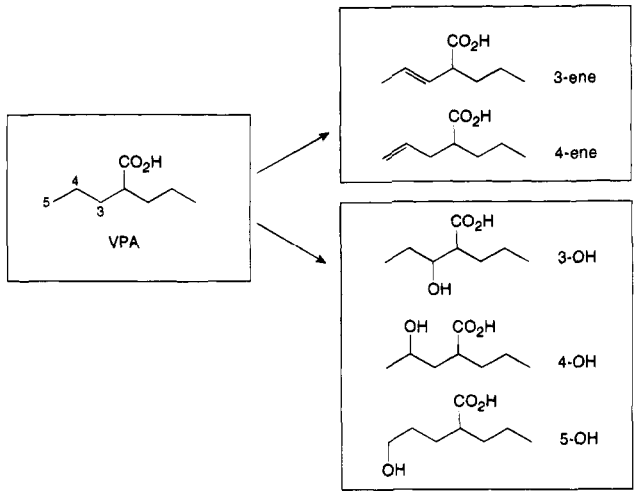

FIGURE 1: Microsomal metabolites of valproic acid.

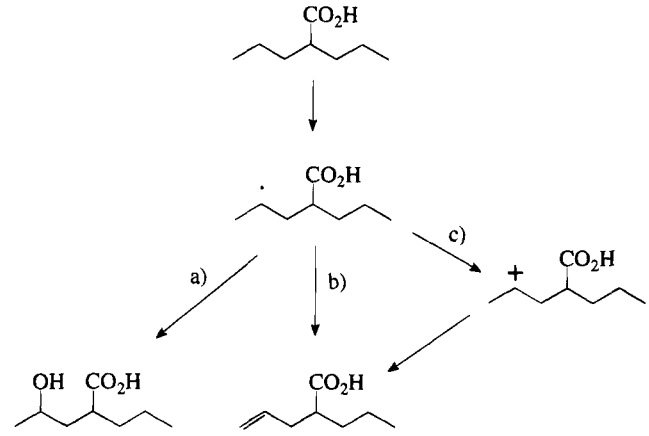

FIGURE 2: Potential fates for a C-4 radical formed during P450-mediated metabolism of VPA.

hypotheses have been advanced for the mechanism by which VPA disrupts fatty acid utilization, including the potential interaction of a reactive metabolite of VPA with one or more enzymes in the mitochondrial β-oxidation sequence (Baillie, 1988). However, while 4-ene-VPA has been shown to be a much more potent cytotoxin, hepatotoxin, and inhibitor of β-oxidation than the parent drug (Kingsley et al., 1983; Kesterson et al., 1984; Bjorge & Baillie, 1985), a specific role for 4-ene-VPA in the etiology of this idiosyncratic reaction remains circumstantial (Schulz, 1991). From the data presently available it seems likely that the hepatotoxic effects of 4-ene-VPA are a result of its further biotransformation to chemically reactive intermediates (Baillie, 1988; Kassahun et al., 1994).

The *N*-acetylcysteine conjugate of (*E*)-2,4-diene-VPA has been identified in the urine of humans administered VPA (Kassahun et al., 1991). This conjugate presumably arises from catabolism of the glutathione conjugate of the diene, which has been characterized as a biliary metabolite of 4-ene-VPA in the rat (Kassahun et al., 1994). This glutathione conjugate most likely is derived from the electrophilic CoA thioester of (*E*)-2,4-diene, which in turn, could arise directly by β-oxidation of 4-ene-VPA or, indirectly, by P450-mediated desaturation of 2-ene-VPA (Kassahun & Baillie, 1993). In either case, P450-mediated terminal desaturation assumes a pivotal role in the overall bioactivation process.

Critical questions remain to be answered regarding both the nature of the P450 isoforms which catalyze terminal desaturation of VPA and the mechanism of this bioactivation reaction. Although CYP2B isoforms in the rat (Rettie et al., 1987) and rabbit (Rettie et al., 1988) are capable of supporting the reaction, specific activities are too low to permit a detailed study of the reaction mechanism with these purified proteins. Consequently, the single mechanistic study conducted to date used crude microsomal mixtures as the enzyme source to evaluate the magnitude of the intramolecular deuterium isotope effects on the formation of 4-ene-VPA derived from [4,4-$^2H_2$]VPA and [5,5,5-$^2H_3$]VPA (Rettie et al., 1988). The results from this study were interpreted in favor of a mechanism which involves initial hydrogen atom abstraction from the ω-1 position to generate the C-4 radical (Figure 2). Subsequent branching between oxygen rebound (pathway a) and hydrogen atom removal (pathway b) could yield 4-hydroxy-VPA or 4-ene-VPA, respectively (Rettie et al., 1988). Alternatively, it has been proposed that desaturation could proceed from a carbocation intermediate (pathway c), which would yield the terminal olefin upon deprotonation (Ortiz de Montellano, 1989).

In order to resolve these mechanistic questions it is clearly important to have available a pure, high specific activity VPA 4-ene, desaturase. In preliminary experiments we found that NADPH-supplemented lung microsomes from untreated rabbits exhibited high rates of formation of 4-ene-VPA. Unexpectedly, this enzyme preparation also formed relatively large amounts of 5-hydroxy-VPA. Therefore, the principal objectives of this study were (i) to identify the lung P450 isoform responsible for the high rates of 4-ene-VPA formation and (ii) to elucidate the relationship between ω-hydroxylation and terminal desaturation of VPA.

EXPERIMENTAL PROCEDURES

Chemicals. VPA and 1-methyl-1-cyclohexanecarboxylic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). An ethanolic solution of VPA was hydrogenated over 10% palladium/charcoal prior to use. Lauric acid (dodecanoic acid), 12-hydroxylauric acid, NADPH, and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). 15-Hydroxypentadecanoic acid was purchased from Wiley Organics (Coshocton, OH). *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Supelco, Inc. (Bellefonte, PA). 4-Hydroxy-VPA, 5-hydroxy-VPA, 4-ene-VPA, 3-ene-VPA, and 3-hydroxy-VPA were obtained as described previously (Rettenmeier et al., 1985, 1986). The synthesis of [4,4-$^2H_2$]VPA and [5,5,5-$^2H_3$]VPA has also been described previously (Rettie et al., 1988).

Instrumentation. Quantitative GC/MS analyses of the trimethylsilyl (TMS) derivatives of oxidized valproic acid metabolites were performed on a VG 7070H double-focusing mass spectrometer interfaced to an HP5710A gas chromatograph. Analyses were performed in the electron impact mode at an electron energy of 70 eV, a trap current of 100 μA, and an accelerating voltage of 4 kV. The temperatures of the ion source and the GC interface were maintained at 200 and 250 °C, respectively. Quantitative GC/FID analyses of the TMS derivative of 12-hydroxylauric acid were performed on an HP 5890 gas chromatograph equipped with an HP3396A integrator. Helium was used as the carrier gas at a head pressure of 15 psi. Injector and detector temperatures were held at 250°C.

Enzyme Sources. Male and female New Zealand White rabbits (2−3kg) were obtained from R&R Laboratories (Bellevue, WA). Lung homogenates (10% w/v) were prepared in 10 mM potassium phosphate, 10 mM EDTA and 0.15 M KCl, pH 7.4, using a Waring blender operated at full power for two 10-s bursts. The homogenate was filtered through two layers of gauze, and microsomes were prepared by differential centrifugation as described previously (Rettie et al., 1989). The initial microsomal pellets were resuspended in 0.1 M pyrophosphate buffer, pH 7.4, and centrifuged again at high speed. Washed microsomal pellets were stored in small aliquots at a protein concentration of 10−15 mg/mL at −80 °C in 0.1 M potassium phosphate buffer, pH 7.4. Specific contents were typically 0.25−0.35 nmol of P450/mg of protein.

CYP4A1, CYP4B1, and CYP4A3 were expressed in HepG2 cells as previously described (Aoyama et al., 1990; Gonzalez et al., 1991). Cells were lysed, and a particulate fraction was prepared for metabolic studies by centrifugation of the lysate at 180000g × 60 min. Specific contents of the cDNA-expressed enzymes were 50−80 pmol of P450/mg of protein.

CYP2B1 (15 nmol/mg) and NADPH−cytochrome P450 reductase (11 nmol/mg) were purified to apparent electrophoretic homogeneity from phenobarbital-pretreated Sprague−Dawley rats, as previously described (Rettie et al., 1987). Cytochrome b_5 purified from rat liver was a gift from Dr. K. E. Thummel (Department of Pharmaceutics, University of Washington).

Metabolic Reaction Conditions. Microsomal incubations were carried out for 30−60 min at 37 °C, in a final volume of 1.0 mL containing 100 μmol of potassium phosphate, pH 7.4, 1 μmol of substrate, (VPA, [4,4-$^2H_2$]VPA, [5,5,5-$^2H_3$]-VPA, or lauric acid), 1 μmol of NADPH, 1 μmol of NADH, and 3 μmol of $MgCl_2$. Incubation mixtures also contained either 300−500 pmol of rabbit lung microsomal P450 or 80−150 pmol of the individual cDNA-expressed CYP4 isoforms. Incubation mixtures were equilibrated for 2 min at 37 °C, prior to initiation of reaction with substrate (10 mM solution in 0.1 M phosphate buffer, pH 7.4). Reactions were terminated by the addition of 1 mL of 10% HCl.

Reconstitution Conditions for Purified CYP2B1. Purified CYP2B1 (1 nmol), P450 reductase (1.1 nmol), and dilaurylphosphatidylcholine (30 μL of a 1 mg/mL aqueous, sonicated suspension) were preincubated at room temperature for 5 min prior to the addition of cytochrome b_5 (1 nmol). This reconstituted enzyme mixture was then incubated as above for native or cDNA-expressed microsomal enzymes in a final volume of 1.0 mL containing 100 μmol of potassium phosphate, pH 7.4, 1 μmol of substrate, (VPA, [4,4-$^2H_2$]VPA, [5,5,5-$^2H_3$]VPA, or lauric acid), 1 μmol of NADPH, 1 μmol of NADH, and 3 μmol of $MgCl_2$. CYP2B1 reactions were terminated after 60 min with 1 mL of 10% HCl.

Antibody Inhibition. To investigate the involvement of specific P450 isoforms in the formation of 4-ene-VPA, 4-hydroxy-VPA, and 5-hydroxy-VPA by rabbit lung microsomes, microsomal preparations were preincubated with polyclonal antibodies (0−15 mg of IgG/nmol of total microsomal P450) for 30 min on ice. Samples were then preequilibrated at 37 °C for 2 min prior to the addition of NADPH and substrate. Reactions were allowed to proceed for 30 min and were terminated with acid, as described above.

General Analytical Procedures. After the addition of internal standards (0.19 μg of 1-methyl-1-cyclohexanecarboxylic acid for VPA samples and 1.6 μg of 15-hydroxypentadecanoic acid for lauric acid samples), the acidified metabolic incubation mixtures were centrifuged (2000g × 10 min) to sediment precipitated proteins. The supernatants were extracted with ethyl acetate (2 × 3 mL). The combined organic extracts were dried with anhydrous $MgSO_4$, and their volume was reduced to approximately 75 μL under a stream of dry nitrogen. Each concentrated organic extract was derivatized with 50 μL of BSTFA at 90 °C for 45 min. The derivatized samples were stored in tightly capped ReactiVials at −20 °C prior to analysis by selected ion monitoring GC/MS or GC/FID.

Analysis of VPA Metabolites. Gas chromatographic separation of derivatized VPA metabolites was carried out on a fused silica capillary column (60 m × 0.32 mm i.d. × 0.25 μm film thickness) coated with DB-1 stationary phase (J&W Scientific, Folsom, CA). Samples were injected in the splitless mode at an initial oven temperature of 40 °C. After 0.5 min, the temperature was raised rapidly to 80 °C, and thereafter at 2 °C/min until the temperature reached 105 °C in order to elute VPA and its unsaturated metabolites. Hydroxylated metabolites of VPA were eluted with a linear temperature gradient of 10 °C/min from 105 to 250 °C. The retention times of the oxidized metabolites of VPA (following derivatization where appropriate) were as follows: (2*R*,4*S*/2*S*,4*R*)-4-hydroxy-VPA-γ-lactone, 11 min 55 s; (2*R*,4*R*/2*S*,4*S*)-4-hydroxy-VPA-γ-lactone, 12 min 10 s; 4-ene-VPA, 12 min 25 s; 3-ene-VPA, 12 min 55 s; 3-hydroxy-VPA, 20 min 30 s; 5-hydroxy-VPA, 22 min 5 s. The TMS derivative of the internal standard, 1-methyl-1-cyclohexanecarboxylic acid, eluted at 14 min 5 s.

The following ions were used for GC/MS analyses of metabolic incubations conducted with unlabeled VPA: *m/z* 199 ($[M-CH_3]^+$ of 4-ene-VPA TMS, 3-ene-VPA TMS, and 1-methyl-1-cyclohexanecarboxylic acid TMS), 100 ($[M-C_3H_6]^{\bullet+}$ of 4-hydroxy-VPA-γ-lactone), 131 ($[C_2H_5CHO-TMS]^+$ of 3-hydroxy-VPA bis[TMS]), and 185 ($[M-C_2H_5-TMS-OH]^+$ of 5-hydroxy-VPA bis[TMS]). Quantitation was achieved by measuring metabolite:internal standard ion ratios and comparing these values to standard curves prepared by extracting known quantities of the authentic synthetic standards from microsomal incubations which lacked NADPH, treating the extracts with BSTFA, and analyzing by GC/MS as outlined above.

Analysis of Lauric Acid Metabolites by GC/FID. Derivatized lauric acid metabolites were also separated on a fused silica capillary column (60 m × 0.32 mm i.d. × 0.25 μm film thickness) coated with DB-1. Samples were injected at an initial oven temperature of 100 °C. After 0.5 min, the temperature was raised rapidly to 160 °C and thereafter at 4 °C/min to 210 °C, and then at 10 °C/min to 290 °C. The retention times of the bis[TMS] derivatives of 12-hydroxylauric acid and 15-hydroxypentadecanoic acid under these conditions were 16.3 and 22.5 min, respectively. Quantitation was achieved from a comparison of metabolite and internal standard peak areas.

Calculation of k_H/k_D. Intramolecular isotope effects on the formation of 4-ene-VPA, 4-hydroxy-VPA, and 5-hy-

Table 1: Regioselective Formation of Oxidized Valproic Acid Metabolites Generated from Rabbit Lung Microsomes

metabolite	rates of formation (nmol/nmol of P450/30 min)
3-ene-VPA	<0.3
4-ene-VPA	3.9 ± 0.35
3-hydroxy-VPA	<3
4-hydroxy-VPA	15 ± 1.1
5-hydroxy-VPA	501 ± 84

droxy-VPA from [4,4-$^2H_2$]VPA and [5,5,5-$^2H_3$]VPA were calculated as detailed previously (Rettie et al., 1988).

Other Procedures. Protein concentrations were determined by the method of Lowry et al. (1951). P450 concentrations were estimated by the method of Estabrook et al. (1972). Kinetic analyses were performed with the k•cat program (Biometallics Inc., Princeton, NJ) which fits velocity−substrate concentration data to a nonlinear kinetics program.

RESULTS

Identification of CYP4B1 as a Microsomal Desaturase of Valproic Acid. Table 1 shows the rates of formation of several VPA metabolites generated by NADPH-supplemented rabbit lung microsomes. Only 4-ene-VPA, 4-hydroxy-VPA, and 5-hydroxy-VPA were detected. 3-ene-VPA and 3-hydroxy-VPA were not formed in significant amounts in these incubations. The rate of formation of 4-ene-VPA (3.9 ± 0.35 nmol/nmol/30 min) in these pulmonary preparations is the highest yet found for this microsomal desaturation reaction. Interestingly, rabbit lung microsomes functioned predominantly as a VPA ω-hydroxylase, and formed 5-hydroxy-VPA with a turnover number of 16.7 min^{-1}.

Rabbit lung microsomal P450 is composed largely of two isoforms, CYP2B4 and CYP4B1 (Serabjit-Singh et al., 1979). Figure 3 illustrates the effects of polyclonal antibodies directed against CYP2B4 and CYP4B1 on the formation of metabolites of VPA by rabbit lung microsomes. These antibodies have been shown previously to be monospecific for the respective rabbit lung P450 isoforms and to maximally inhibit rabbit lung microsomal CYP2B- or CYP4B-dependent catalysis at concentrations of 5 mg of IgG/mg of microsomal protein (corresponding to approximately 15 mg of IgG/nmol of total microsomal P450) (Serabjit-Singh et al., 1979). The rate of formation of 5-hydroxy-VPA in rabbit lung microsomes was reduced to 5% of control levels by anti-4B. Conversely, anti-2B was without effect on the 5-hydroxylation of VPA. This suggests that CYP4B1 is responsible for practically all of the 5-hydroxy metabolite generated by rabbit lung microsomes, whereas CYP2B4 appears to be responsible for the majority of the 4-hydroxylation in lung microsomes. The antibody inhibition profiles suggest further that both CYP2B4 and CYP4B1 contribute to lung microsomal 4-ene-VPA formation, but that CYP4B1 is by far the greater contributor, responsible for approximately 85% of the turnover to this olefinic metabolite.

Table 2: Correlation of VPA 4-ene-desaturase Activity with Microsomal Content of Rabbit CYP4B1

enzyme source[a]	rate of formation (nmol/nmol P450/30min)	CYP4B1 content[b] (%)
UT rabbit liver	0.09 ± 0.01[c]	<2
PB rabbit liver	0.56 ± 0.03[b]	7−15
UT rabbit lung	3.9 ± 0.35	40−50
cDNA-expressed CYP4B1	8.7 ± 0.30	100

[a] UT and PB denote untreated and phenobarbital-pretreated, respectively. [b] Immunochemical data taken from Robertson et al. (1983). [c] Catalytic data taken from Rettie et al. (1988).

CYP4 Isozyme Specificity for Metabolism of Valproic Acid. Confirmation that CYP4B1 is a VPA 4-ene desaturase was obtained from studies with preparations of the enzyme which had been expressed in HepG2 cells. cDNA-expressed CYP4B1 formed 4-ene-VPA in an NADPH-dependent manner at a rate of 8.7 ± 0.30 nmol/nmol of P450/30 min (Table 2), which is approximately 60 times faster than was found previously with purified and reconstituted CYP2B1 and CYP2B4 (Rettie et al., 1987, 1988). Rates of formation of 4-ene-VPA correlated well with the previously published

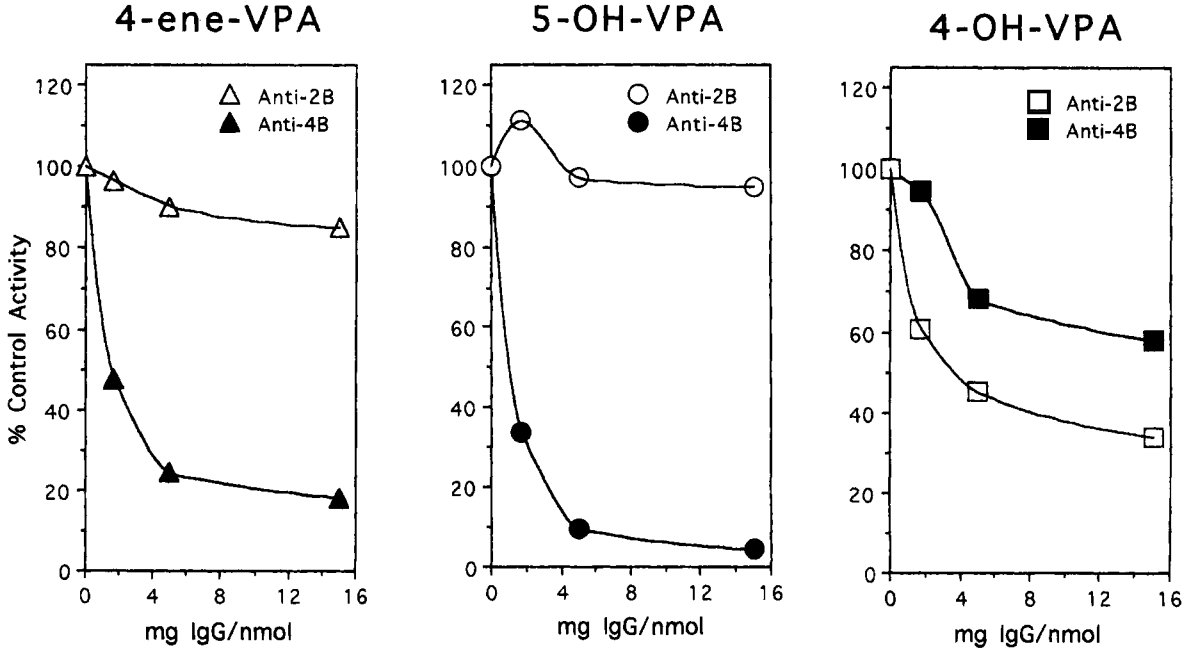

FIGURE 3: Antibody inhibition titration curves for the formation of VPA metabolites by rabbit lung microsomes.

Table 3: CYP4 Isoform Selectivity for Metabolism of Valproic Acid

	rate of metabolite formation (nmol/nmol of P450/min)			
isoform	4-ene-VPA	4-hydroxy-VPA	5-hydroxy-VPA	ω:ω-1
CYP4A1[a]	<0.01	<0.1	<0.05	
CYP4A3[a]	<0.01	<0.1	<0.05	
CYP4B1	0.29 ± 0.0	0.69 ± 0.14	34 ± 7.1	49:1

[a] These preparations of CYP4A1 and CYP4A3 catalyzed the 12-hydroxylation of lauric acid at rates of 23 and 7.3 nmol/nmol of P450/min, respectively, as determined by GC/FID analysis of the bis(trimethylsilyl) derivative, as described in Materials and Methods.

Table 4: Intramolecular Deuterium Isotope Effects on the Formation of VPA Metabolites by CYP2B1 and CYP4B1

		k_H/k_D	
substrate	metabolite	CYP2B1	CYP4B1
[4,4-$^2H_2$]VPA	4-ene-VPA	3.6 ± 0.12	7.6 ± 0.41
	4-hydroxy-VPA	3.7 ± 0.29	>5[a]
	5-hydroxy-VPA	1.0 ± 0.05	1.3 ± 0.01
[5,5,5-$^2H_3$]VPA	4-ene-VPA	1.2 ± 0.12	1.8 ± 0.02
	4-hydroxy-VPA	1.2 ± 0.04	1.3 ± 0.07
	5-hydroxy-VPA	6.4 ± 0.98	4.4 ± 0.02

[a] Accurate measurement precluded by the low abundance of the ion currents at the diagnostic *m/z* values of 127 and 128 ([M-CH_3]$^+$ for $^1H_2$ and $^2H_2$ forms, respectively, of the the lactone generated from 100 pmol of CYP4B1.

(Robertson et al., 1983) immunochemically determined content of rabbit CYP4B1 from a variety of enzyme sources (Table 2).

cDNA-expressed CYP4B1 hydroxylated VPA with a high degree of regioselectivity for the ω carbon (Table 3). The turnover number for formation of 5-hydroxy-VPA was estimated to be 34 min^{-1}, and the ω:ω-1 hydroxylation ratio was 49:1. CYP4B1 proved to be a relatively inefficient ω-1 hydroxylase of VPA, exhibiting a turnover to the 4-hydroxy metabolite only 2−3 times greater than that for terminal desaturation. This observation is significant because it suggests a heretofore unsuspected association between ω-hydroxylation of VPA and terminal desaturation. In an attempt to probe this relationship further, we examined the metabolism of VPA by the two lauric acid ω-hydroxylases CYP4A1 and CYP4A3, also expressed in HepG2 cells. However, no metabolites of VPA above background levels were detected in incubations of VPA with either CYP4A1 or CYP4A3, although parallel incubations with lauric acid confirmed the metabolic competence of these two enzyme preparations.

Deuterium Isotope Effect Studies for CYP2B1- and CYP4B1-Mediated Metabolism of VPA. Intramolecular deuterium isotope effects for the CYP4B1- and CYP2B1-dependent formation of 4-ene-VPA, 4-hydroxy-VPA, and 5-hydroxy-VPA are shown in Table 4. Isotope effects of 3.6−7.6 were obtained for 4-ene-VPA derived from [4,4-$^2H_2$]VPA. Similarly large isotope effects were obtained for 4-hydroxy-VPA generated from [4,4-$^2H_2$]VPA by either CYP2B1 or CYP4B1. As expected, low secondary isotope effects of 1−1.3 were observed for formation of the 5-hydroxy metabolite from [4,4-$^2H_2$]VPA with both CYP2B1 and CYP4B1.

Isotope effects of 4.4−6.4 were observed for 5-hydroxy-VPA formation from [5,5,5-$^2H_3$]VPA, whereas the observed isotope effect for desaturation of [5,5,5-$^2H_3$]VPA to 4-ene-VPA was less than 2. Again, the isotope effects for 4-ene-VPA and 4-hydroxy-VPA were seen to cosegregate. Table 5 contrasts the positional specificity of VPA metabolism by CYP2B1 and CYP4B1. CYP2B1 can be seen to function principally as an ω-1 hydroxylase of VPA, whereas CYP4B1 is clearly an ω-hydroxylase of VPA. Therefore, collectively, these data suggest that the rate-limiting step in P450-catalyzed formation of 4-ene-VPA is hydrogen atom abstraction from the C-4 position, *regardless of the regioselectivity of the isoform involved.*

Table 5: Product Distribution and Partition Ratios for VPA Metabolites Generated from CYP2B1 and CYP4B1

	rate of metabolite formation[a] (nmol/nmol/30 min)	
	CYP2B1	CYP4B1
product distribution		
4-ene-VPA	0.18 ± 0.01	8.7 ± 0.30
4-hydroxy-VPA	6.6 ± 0.50	21 ± 4.2
5-hydroxy-VPA	0.85 ± 0.05	1020 ± 210
partition ratios		
C-4 hydroxylation:desaturation	37:1	2:1
C-4 hydroxylation:C-5 hydroxylation	8:1	0.02:1

[a] Rates of metabolite formation are the mean ± SD of 3 or 4 determinations, from reactions conducted with unlabeled VPA.

Influence of C-5 Hydroxylation on the Partition between Desaturation and C-4 Hydroxylation of VPA. The C-4-centered radical, common to both CYP2B1- and CYP4B1-dependent formation of 4-ene-VPA, may partition between the oxygen rebound product, 4-hydroxy-VPA, and the desaturated product, 4-ene-VPA (see Figure 2). If events subsequent to abstraction of the initial hydrogen atom from C-4 played no role in determining the formation of 4-ene-VPA, then rates of formation of the terminal olefin should be proportional to the rate of 4-hydroxylation for a given isoform, and the partition ratio between C-4 hydroxylation and terminal desaturation would be a constant. However, the data summarized in Table 5 show that this partition ratio is 2:1 for CYP4B1 and 37:1 for CYP2B1. Clearly, events following the initial hydrogen atom abstraction from C-4 do modulate the extent of terminal desaturation, and comparison of the positional specificities for VPA hydroxylation catalyzed by CYP2B1 and CYP4B1 (Table 5) suggests that facile ω-hydroxylation is an important factor.

DISCUSSION

A primary aim of the studies reported in this paper was to identify the high specific activity 4-ene-VPA desaturase present in rabbit tissues. We had suggested earlier that CYP4B1, or cytochrome P450 LM5 as it was termed previously, may be responsible for elevated rates of formation of 4-ene-VPA observed with liver microsomes from rabbits induced with phenobarbital (Rettie et al., 1988). High rates of formation in rabbit lung microsomes are consistent with this suggestion since both enzyme sources are rich in CYP4B1 (Robertson et al., 1983). Unfortunately, however, CYP4B1 is not readily isolated in large quantities in a catalytically competent manner, and so we chose, initially, to examine the effect of monospecific inhibitory antibodies directed against CYP4B1 on the metabolism of VPA by rabbit lung microsomes, which are a rich source of this isoform (Philpot et al., 1991). The results of these experiments demonstrate clearly that CYP4B1 catalyzes the majority of 4-ene-VPA formation in rabbit lung microsomes.

Additional experiments performed with the cDNA-expressed enzyme confirmed that CYP4B1 is a highly effective VPA 4-ene desaturase.

Purified CYP4B1 has been shown to hydroxylate lauric acid at the ω-position, but without a high degree of regioselectivity (ω:ω-1 = 1.4−1.6) (Williams et al., 1984; Muerhoff et al., 1989) In contrast, CYP4B1 is a highly selective ω-hydroxylase of VPA (ω:ω-1 = 49). What then is the relationship between terminal desaturation and ω-hydroxylation of VPA? To address this question, we first attempted to measure rates of terminal desaturation of VPA by the classical lauric acid ω-hydroxylases, CYP4A1 and CYP4A3. However, these isoforms did not metabolize VPA. Therefore, we turned our attention to intramolecular deuterium isotope effect studies on the formation of VPA metabolites, using two P450 isoforms with very different regioselectivities for ω and ω-1 hydroxylation of VPA.

Since the magnitude of an observed isotope effect for P450-mediated oxidation can be influenced greatly by the regioselectivity of the isoform(s) involved (Jones et al., 1986, 1990; Singh et al., 1992), it is important to conduct mechanistic studies with pure isoforms where possible and to interpret the data in light of the isoform's positional specificity for hydroxylation. Consequently, we performed these studies with cDNA-expressed CYP4B1 and purified reconstituted CYP2B1 and determined the magnitude of the observed isotope effects on each of the three metabolites generated by these two isoforms.

As expected, P450-mediated formation of 4-hydroxy-VPA from [4,4-$^2H_2$]VPA and of 5-hydroxy-VPA from [5,5,5-$^2H_3$]-VPA exhibited relatively large isotope effects of 3.7 or greater. The magnitude of the observed isotope effect on CYP4B1-catalyzed ω-hydroxylation from [5,5,5-$^2H_3$]VPA is smaller than the value obtained with CYP2B1. This could be a result of CYP4B1's relative inability to branch from ω to ω-1 hydroxylation upon deuterium substitution at the terminal position. Additionally, since VPA contains a carboxylate group which conceivably could form an ion pair in the enzyme active site, free rotation (and thus interchange) of the protium- and deuterium-containing *n*-propyl side chains may be hindered, thereby masking full expression of the isotope effect.

Formation of 4-hydroxy-VPA from [5,5,5-$^2H_3$]VPA and of 5-hydroxy-VPA from [4,4-$^2H_2$]VPA proceeded with deuterium isotope effects of less than 1.3, also as expected. These isotope effect values serve as internal positive controls for this experiment and permit the isotope effects for the formation of 4-ene-VPA from [4,4-$^2H_2$]VPA and [5,5,5-$^2H_3$]-VPA of 3.6−7.6 and 1.2−1.8, respectively, to be interpreted in favor of rate-limiting hydrogen atom abstraction from the C-4 position.

The partition ratio between oxygen rebound and the second step on the desaturation reaction cooordinate is, therefore, approximately 2:1 for the ω-hydroxylase CYP4B1. The corresponding partition ratio for the ω-1 hydroxylase CYP2B1 is almost 40:1. Consequently, ω-hydroxylation of VPA plays an important role in modulating the flux through the 4-ene pathway during CYP4B1 catalysis, and it appears to do so by influencing events which occur during the second half-reaction. A somewhat analogous situation exists for the CYP2A1-catalyzed formation of 6-ene-testosterone. CYP2A1 is predominantly a 7α-hydroxylase of testosterone (90% of the primary metabolites), but desaturation proceeds via rate-limiting hydrogen atom abstraction from the 6α position (Korzekwa et al., 1990). In this case the partition ratio between oxygen rebound and desaturation is 1:1.

The nature of the second half-reaction in terminal desaturation of valproic acid remains to be elucidated. Two possible mechanisms are depicted in Figure 2. Although the regioselectivity data and deuterium isotope effects presented in this paper do not permit discrimination between deprotonation and hydrogen atom abstraction pathways, the complete absence of 3-ene-VPA formation from either CYP2B1 or CYP4B1 suggests strongly that, if the carbocation pathway is involved, the deprotonation step in the second half-reaction must be enzyme-assisted. Therefore, for either the dual hydrogen atom abstraction mechanism (pathway b, Figure 2) or the deprotonation of a carbocation (pathway c), substrate movement in the active site is required to complete the second half-reaction. This is a recognized phenomenon during P450 catalysis, at least for radical species (White, 1991), and it provides a plausible explanation for the enhanced VPA desaturase activity of CYP4B1, since the product distribution demonstrates that 97% of productive catalytic events arise from orientation of the terminal carbon of VPA over the iron−oxene species of the enzyme.

Finally, the finding that CYP4B1 is an ω-hydroxylase of VPA greatly extends the known substrate specificity of this isoform. Although it has been recognized for some time that CYP4B1 can catalyze hydroxylation of lauric acid, it does so with little regioselectivity and with a relatively low turnover number (Williams et al., 1984). Indeed, few reactions are recognized to be characteristic of CYP4B1, and the focus to date has been on the bioactivation of xenobiotics such as 4-ipomeanol and a number of arylamines (Vanderslice et al., 1987; Czerwinski et al., 1991; Verschoyle et al., 1993). It is tempting to speculate that the high turnover and regiospecificity of the ω-hydroxylation of VPA by rabbit CYP4B1 may be indicative of this isoform's physiological function. Studies are underway to define the substrate specificity of CYP4B1 for short, branched-chain fatty acids.

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