Reassessment of Cytochrome P450 2B2: Catalytic Specificity and Identification of Four Active Site Residues†

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ABSTRACT: Cytochromes P450 2B metabolize a variety of compounds and have provided an excellent framework for identifying determinants of substrate specificity. Among the rat 2B enzymes, a puzzling difference has emerged between the reported substrate specificity of purified hepatic 2B2 and that of certain 2B1 mutants containing 2B1 → 2B2 substitutions. To address these discrepancies, we have characterized two 2B2 variants. A cDNA clone designated $2B2_{FF}$ was obtained from phenobarbital-induced Lewis rats and, like some previously isolated variants, was found to contain phenylalanine at positions 58 and 114. A second 2B2 clone was generated by restoring Leu and Ile, respectively, at these positions. These enzymes were expressed in *Escherichia coli* and analyzed with androstenedione, testosterone, progesterone, ethoxycoumarin, benzyloxyresorufin, and pentoxyresorufin. The expressed 2B2 variants metabolized most substrates at rates that were 1−9% of those of 2B1. When steroid regio- and stereospecificity was examined, the metabolite profiles of expressed 2B2 and $2B2_{FF}$ conflicted with the 16β- and 16α-hydroxylation observed for purified hepatic 2B2 from Sprague-Dawley rats. These and other results suggested that the purified hepatic 2B2 contained a small percent of the 2B1 enzyme. Masses of tryptic peptides were consistent with identity between purified hepatic 2B2 and $2B2_{FF}$. On the basis of a three-dimensional homology model and the construction and analysis of 2B2 mutants, residues 114, 363, 367, and 478 were identified as determinants of substrate specificity. In addition, 2B1 and the expressed 2B2 variants showed differential susceptibility to the mechanism-based inactivators chloramphenicol and *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide.

Cytochromes P450 represent a superfamily of enzymes that are responsible for the metabolism of a multitude of endogenous and exogenous compounds. Eleven P450[1] families and 18 subfamilies have been identified in rats on the basis of the analysis of 44 genes (*1*). Early work on rat P450 enzymes involved the isolation of various inducible isozymes from the liver and characterization of these purified hepatic proteins. Two of these, 2B1 and 2B2, represent 55% of the total P450 content of the liver following induction with phenobarbital (*2*). P450 2B1 and 2B2 differ by 14 amino acids (*3, 4*) and are thought to yield similar steroid metabolites. Thus, both purified hepatic enzymes were found to exhibit 16β- and 16α-hydroxylation of androstenedione and testosterone and 16α-hydroxylation of progesterone, with 2B1 activity being 5−10-fold higher than that of 2B2 (*5*−*8*). This is in stark contrast to the rabbit enzymes 2B4 and 2B5, which differ by 12 residues (*9, 10*) yet exhibit different substrate specificities. In particular, 2B4 is an androstenedione 16β-hydroxylase, while 2B5 is a 16α- and 15α-hydroxylase (*11*). In addition, only 2B5 has appreciable activity toward progesterone (*10*).

More recently, 2B1 expressed in COS or *Escherichia coli* cells has shown activity and substrate specificity comparable to that of the purified hepatic 2B1 protein (*12*−*14*). Through the analysis of 2B1 variants and site-directed mutants, residues 114, 206, 209, 302, 363, 367, 477, 478, and 480 have been identified as determinants of substrate specificity (*12, 13, 15*−*19*). These key residues fall in or near regions proposed by Gotoh to be substrate recognition sites (SRS) based on comparative sequence analysis and analogy to bacterial P450 101 (*20*). Of the 14 amino acid differences between 2B1 and 2B2, three are active site residues, namely 363, 367, and 478. When these residues in 2B1 were altered to those present in 2B2, the resulting mutants exhibited new metabolite profiles or were made refractory to inactivation by known 2B1 inhibitors (*13, 16*−*18*). For example, a 2B1 mutant with a Val → Ala substitution at residue 363 exhibits androstenedione 15α-hydroxylase activity not observed for the wild-type enzyme (*17*). Furthermore, a natural 2B1 variant in Wistar Munich rats that contains a single Ala → Gly substitution at residue 478 is refractory to inactivation by the 2B1 inhibitor *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide (*12, 13*). These studies made it difficult to understand how 2B2 could yield the same steroid metabolites and be inactivated by the same compounds as 2B1 (*21*).

Since previous studies of 2B2 have been based primarily on purified hepatic proteins, we have isolated, heterologously expressed, and characterized a 2B2 cDNA clone. The steroid hydroxylation profiles of this and another expressed 2B2

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[1] Abbreviations: P450, cytochrome P450; SRS, substrate recognition site; PCR, polymerase chain reaction; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DLPC, dilauroyl-L-3-phosphatidylcholine; TLC, thin-layer chromatography; reductase, NADPH−cytochrome P450 reductase.

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variant do not match those of purified hepatic 2B2. These and other results lead us to conclude that the purified hepatic 2B2 protein contains a small amount of the higher-activity 2B1 enzyme along with $2B2_{FF}$. Computer modeling was used to map the amino acid differences between 2B1 and several 2B2 variants and assisted in identifying residues that were involved in substrate specificity. In addition to providing additional information about 2B enzymes, these findings have implications for other studies using proteins purified from tissues, especially when working within a large subfamily of closely related enzymes.

EXPERIMENTAL PROCEDURES

Materials. Male Lewis rats (180−200 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Primers were synthesized by the University of Arizona Macromolecular Structure Facility (Tucson, AZ) or by National Biosciences, Inc. (Plymouth, MN). Restriction endonucleases, *Taq* polymerase, media for bacterial growth, and the 3′ RACE kit were purchased from Gibco-BRL (Grand Island, NY). The Sequenase 2.0 dideoxy sequencing kit was from the United States Biochemical Corp. (Cleveland, OH). The *E. coli* strain Topp3 was from Stratagene (La Jolla, CA). Androstenedione, testosterone, progesterone, resorufin, 7-benzyloxyresorufin, 7-pentoxyresorufin, NADPH, DLPC, and CHAPS were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin was from the Aldrich Chemical Co. (Milwaukee, WI). Hydroxylated metabolites of androstenedione and testosterone were obtained from Steraloids (Wilton, NH). Guanidinium thiocyanate was obtained from Fluka BioChemika (Ronkonkoma, NY). HEPES was purchased from Calbiochem Corp. (La Jolla, CA). [^{14}C]Androstenedione and [^{14}C]progesterone were purchased from DuPont-New England Nuclear (Boston, MA). [^{14}C]Testosterone was purchased from Amersham Life Science, Inc. TLC plates [silica gel, 250 mM, Si 250PA (19C)] were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Rat NADPH−P450 reductase was expressed in *E. coli* and purified as described previously (*22*). All other reagents and supplies were obtained from standard sources.

Isolation of the P450 2B2 cDNA Clone. Two male Lewis rats were given 0.1% sodium phenobarbital (Mallinckrodt, Inc.) in the drinking water for 4 days, followed by untreated drinking water for 1 day. Hepatic RNA was isolated using the guanidinium thiocyanate method (*12*). A hepatic cDNA library was constructed with the 3′ RACE kit, using conditions specified by the manufacturer. The N-terminal (5′-GGACCATGGCTCCCAGTATCTTGC) and C-terminal (5′-CCCGGGCACCTGGCTGCC) primers were designed to amplify the 1.5 kb cDNA of 2B1 or 2B2. Sequence identity between these two cDNAs did not allow for specific amplification of 2B2 cDNA. The N-terminal primer contained an *Nco*I site just upstream of the start codon and an alanine codon (GCT, underlined in the primer sequence) at the second position to improve expression in *E. coli* (*14*). PCR was initiated by the addition of 1 unit of Deep Vent polymerase (New England Biolabs, Inc., Beverly, MA) during a 5 min incubation at 94 °C. This was followed by 4 cycles of 94 °C for 1 min, 47 °C for 30 s, and 72 °C for 2 min, then continued with 25 cycles of 94 °C for 1 min, 59 °C for 30 s, and 72 °C for 2 min, and ended with one 72 °C incubation for 6 min. PCR products of the appropriate size were purified using Gene Clean (Bio 101, La Jolla, CA). PCR products were treated with *Taq* polymerase (2.5 units per reaction incubated at 72 °C for 15 min) to create the one-base overhang for cloning into the pCR2.1 vector (Invitrogen, San Diego, CA). Restriction enzyme digestions were performed to determine which clones contained full length P450 cDNAs, and digestion with *Bgl*I was used to distinguish 2B2 clones which lacked the *Bgl*I site from 2B1 clones which contained the *Bgl*I site. Two full length 2B2 cDNA clones were sequenced by the University of Arizona Macromolecular Structure Facility or by double-stranded sequencing using Sequenase. The 2B2 cDNA was subcloned into pSE380 (Pharmacia Biotech Inc.) for expression in *E. coli*. To allow for the construction of 2B2 mutants, some of the polylinker sites in pSE380 were eliminated by digestion with *Sal*I and *Xho*I and subsequent ligation to delete the intervening 252 base pairs of the sequence. The 1.5 kb *Nco*I-*Eco*RI fragment from pCR2.1-2B2 was ligated into pSE380(Δ*Sal*I-*Xho*I) digested with the same restriction enzymes and treated with alkaline phosphatase (Boehringer Mannheim), thus generating $pSE2B2_{FF}$.

Construction of 2B2 Mutants. To replace codons 58 and 114 of the variant $2B2_{FF}$ with the 2B1 sequence, the *Nco*I/*Bam*HI insert (encoding residues 1−165) from pKK2B1 (*14*) was ligated into $pSE2B2_{FF}$ treated with the same restriction enzymes, generating pSE2B2. There are no differences in amino acids between 2B1 and the first reported 2B2 in this region (*3, 4*). Construction of mutations at codons 363 and 367 was done in two steps. (1) The *Bgl*II fragment encoding residues 361−486 was isolated from 2B1 cDNA clones [wild type to replace both codons, 2B1 V363A[2] to replace codon 367, or 2B1 V367L to replace codon 363 (*17, 18*)] and ligated into pSE2B2 digested with *Bgl*II and treated with alkaline phosphatase. (2) The *Nco*I/*Kpn*I fragment encoding residues 1−380 (from step 1) was then ligated into pSE2B2 treated with the same enzymes to restore the 2B2 sequence downstream of the region encoding residue 381. The net result was incorporation of the region encoding residues 363 and 367 (within the 60 bp from the first *Bgl*II site to the *Kpn*I site) from 2B1 into 2B2. There are no other amino acid differences between 2B1 and 2B2 in this region. To alter codon 478, PCR was used with the N-terminal primer (5′-GTTCTACCGCAGTTTTCCCTCC) and the C-terminal primer (5′-GAAGATCTGGTACGTTGGAGGTATTTTTCCAATGCCACTC) designed to amplify the region encoding residues 202−487. Following cloning into pCR2.1, the *Bgl*II fragment was subcloned into pSE2B2, as described above. Sequencing confirmed the presence of a glycine codon (GGA, complementary sequence is underlined in the primer) at position 478 and wild-type sequence in the rest of the insert. Double and triple mutants at residues 363, 367, and 478 were constructed as described above, by subcloning the appropriate *Nco*I/*Kpn*I fragment into pSE2B2 A478G that was digested with the same restriction enzymes. All clones were sequenced to verify the presence of mutations and to confirm that no alterations were introduced in the regions of the restriction sites used during cloning.

Expression of P450 2B2 Enzymes in E. coli and Steroid Hydroxylation by P450 2B1 and 2B2 Enzymes. The vector

[2] 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. The triple mutant 2B2 A363V/L367V/A478G is designated 2B2 VVG, and the triple mutant 2B1 V363A/V367L/G478A is designated 2B1 ALA.

pSE380 was used for heterologous expression of 2B2 cDNA clones in *E. coli*. Conditions for expression were as described previously (*14, 18*). Briefly, isopropyl 1-thio-β-D-galactopyranoside (IPTG, final concentration of 1 mM) was added when cells reached an OD_{550} of 1.2−1.5. δ-Aminolevulinic acid (80 mg L^{-1}) was added 1.5−2 h prior to addition of IPTG. The total P450 concentration was measured by reduced CO difference spectra (*23*), and expression levels of P450 were determined by measuring this spectra from sonicated whole-cell lysates. CHAPS-solubilized membrane preparations and enzyme reconstitution were performed as described (*14, 24*). Androstenedione, testosterone, and progesterone hydroxylation activities were measured as described previously (*10, 14, 18*). Each reaction mixture contained 10 pmol of P450, 20 pmol of reductase, 20 pmol of rat liver cytochrome b_5, and 1 mM NADPH in 50 mM Na^+-HEPES buffer at pH 7.6. Substrate concentrations for the ^{14}C-labeled steroids were 25 μM for androstenedione and progesterone and 200 μM for testosterone. The 2B2 enzymes were typically incubated with substrate for 20−30 min, compared with 5−10 min incubations for the higher-activity 2B1 enzyme (see figure legends for incubation times of specific experiments). 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, 6β-OH, and 7α-OH metabolite separation, as described previously (*21*). In addition, the 7α-OH and 6β-OH androstenedione metabolites were confirmed by comigration with the standards on TLC plates. The 15α-OH androstenedione product was enzymatically converted to 15α-OH testosterone using β-hydroxysteroid dehydrogenase (Sigma Chemical Co.) in the presence of NADH and confirmed by TLC comigration with the 15α-OH testosterone standard (*11*). Testosterone metabolites were confirmed by comigration with standards, as previously described (*10*). Progesterone 16α-OH and 6β-OH metabolites were confirmed by comigration with standards in two different chromatography systems [10:1:1 benzene/ethyl acetate/acetone (v/v/v) and 16:8:1 ethyl acetate/*n*-hexane/acetic acid (v/v/v)].

Metabolism of Other Substrates by P450 2B2 and 2B1 Enzymes. Benzyloxyresorufin and pentoxyresorufin *O*-dealkylase activities were measured using fluorometric analysis (excitation at 550 nm and emission at 585 nm), as was 7-ethoxycoumarin deethylase activity (excitation at 366 nm and emission at 454 nm), as previously described (*18, 24, 25*). Reaction mixtures contained 20 pmol of P450, 40 pmol of reductase, and 20 pmol of rat liver cytochrome b_5. Reconstituted enzymes were incubated with substrate for 10 min (2B1) or 20 min (2B2).

Mechanism-Based Inactivation. Inactivation studies were performed as described previously (*12, 13*). The inhibitors chloramphenicol and *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide (*26*) were added from a methanol stock solution to a final concentration of 50 or 250 μM. P450 2B1 was assayed for residual enzyme activity following incubation with inhibitor using 7-ethoxycoumarin as a substrate and 10 pmol of P450 per time point. Inactivation of $2B2_{FF}$ was assayed using either 7-ethoxycoumarin or [^{14}C]progesterone as a substrate, with 20 pmol of P450 per time point. For progesterone, the remaining 16α-hydroxylase activity was measured. Expressed 2B2 enzyme inactivation was assayed using [^{14}C]testosterone as a substrate, with 10 pmol of P450 per time point, and the residual 15α-hydroxylase activity was determined. Ratios of P450:reductase:rat liver cytochrome b_5 of 1:2:2 or 1:2:1 were used for 2B1 and the expressed 2B2 variants, respectively. Following preincubation for 1, 3, 5, 7, or 9 min in the presence of the inhibitor (or methanol for the control), reactions proceeded in the presence of substrate for 10 min (2B1 and $2B2_{FF}$) or 20 min (2B2). Rate constants for inactivation were calculated by linear regression analysis of the natural logarithm of the residual activity as a function of time. The extent of reversible (competitive) inhibition was estimated from the decrease in the extrapolated activity at zero preincubation time compared with the methanol control.

HPLC−Mass Spectrometry and Tandem Mass Spectrometry Analyses. Purified hepatic P450 2B1 and 2B2 proteins used in these studies were isolated previously from Sprague-Dawley rats (*27, 28*). Each P450 protein (5 nmol) was dialyzed for 3 h against water by microdialysis. Dialyzed proteins were digested with modified trypsin for 19 h at 37 °C in a bicarbonate buffer at a ratio of 1:50 (trypsin:P450, w/w). The tryptic fragments were separated by HPLC using a Vydac 250 mm × 4 mm C-18 peptide column, with a linear gradient of H_2O (0.1% TFA) to acetonitrile (0.085% TFA) over 90 min at a flow rate of 1.0 mL min^{-1}. Detection was by UV at 215 nm and positive electrospray mass spectrometry with a Finnigan TSQ 7000 tandem mass spectrometer (San Jose, CA) coupled to a Hewlett-Packard 1050 HPLC instrument (Palo Alto, CA). The tryptic fragments of interest were sequenced using positive ion electrospray tandem mass spectrometry with collision-induced dissociation (CID). Argon was used as the CID gas at a pressure of 1 mTorr and an energy of −30 eV.

Computer Modeling. A three-dimensional model of cytochrome P450 $2B2_{FF}$ was constructed by alteration of the 2B1 model (*19*). INSIGHTII software (Biosym Technologies, San Diego, CA) was used to display structures on a Silicon Graphics workstation. Modifications of the 2B1 model to construct the $2B2_{FF}$ model were performed as described (*10*). The 14 amino acid residues that vary between 2B1 and $2B2_{FF}$ were replaced using the BIOPOLYMER program, and the regions of amino acid replacement were minimized using the DISCOVER program with a consistent valence force field. The steepest descent method and conjugate gradients were used as described previously (*19, 29*).

RESULTS

Isolation and Expression of a P450 2B2 cDNA Clone. A cDNA library was prepared from Lewis rat hepatic RNA, and 2B1/2B2 clones were amplified by PCR. 2B2 cDNA clones were identified on the basis of restriction digestion analysis. Two clones obtained from independent PCRs were sequenced, and the predicted amino acid sequence revealed 14 differences compared with that of 2B1 (*3*): L58F, I114F, S303G, A321T, L337P, T339S, S344T, S360A, V363A, V367L, S407T, N417D, A419T, and G478A. Compared with the sequence of the first reported 2B2 clone (*4*), the cDNA clone had 4 alterations: L58F, I114F, V322E, and M473K. This clone is designated $2B2_{FF}$ to represent the L58F and I114F alterations. Similar 2B2 variants have been isolated previously from Sprague-Dawley rats. Thus, $2B2_{FF}$

has the same predicted protein sequence as that reported by Aoyama et al. (*15*), with the exception of residue 473. In addition, the nucleotide sequence of the $2B2_{FF}$ cDNA clone was identical to a partial clone starting at the codon for residue 105 (*30, 31*). Sequence analysis confirmed that four different 2B2 cDNA clones contained phenylalanine codons at positions 58 and 114.

Expression of P450 2B2 in Topp3 cells typically yielded 50−100 nmol of solubilized P450 per liter of culture after 60 h of induction, with a final recovery of 20−50 nmol of P450 per liter of culture. The expression and recovery of most of the mutant 2B2 enzymes were also within this range. However, Topp3 cells did not provide good recovery of the triple mutant 2B2 VVG. The CHAPS-solubilized membrane preparations from Topp3 cells gave a reduced CO difference spectra with a large shoulder extending from the 450 nm peak. The expression and recovery of 2B2 VVG were improved in DH5α cells, so this strain and a 72 h induction time were used for 2B2 VVG expression. DH5α cells have also been used for the expression of P450 3A4 (*22*). The recovery of 2B2 VVG from DH5α cells was 10 nmol of P450 per liter of culture.

Activity of E. coli-Expressed P450 2B2 Compared with E. coli-Expressed P450 2B1 and Purified Hepatic P450 2B2. CHAPS-solubilized membranes expressing $2B2_{FF}$ exhibited very low androstenedione hydroxylase activity, producing minor amounts of 7α-OH and 15α-OH metabolites (Figure 1, and Table 1). This is contrary to the pattern observed for purified hepatic P450 2B2, where the same androstenedione 16*β*-OH and 16α-OH metabolites as the higher-activity 2B1 enzyme were observed (Figure 1 and refs 5−7). To determine whether the differences in the metabolite profile were due to the presence of phenylalanine at positions 58 and 114, these residues were replaced with the leucine and isoleucine that are present in both 2B1 and the first reported 2B2 (*3, 4*). This expressed 2B2 exhibited androstenedione hydroxylase activity 8-fold higher than that of $2B2_{FF}$ and yielded 15α- and 6*β*-OH products (Figure 1 and Table 1).[3] The metabolite profile of expressed 2B2 was also in contrast with the predominant 16-OH products observed for purified hepatic 2B2. Neither expressed 2B2 variant metabolized androstenedione well, exhibiting only 1−5% of the total activity of 2B1.

Consistent with previous reports of purified hepatic enzymes (*5*−*7*), the purified hepatic 2B2 exhibited androstenedione 16*β*- and 16α-hydroxylation rates that were 10-fold lower than those of 2B1 (Table 1). In addition, minor amounts of 7α-OH and 15α-OH androstenedione were produced at rates that were closer to those of 2B1. Thus, the metabolite profile of purified hepatic 2B2 appeared to be a combination of the $2B2_{FF}$ and 2B1 activities. Two different purified hepatic 2B2 preparations from Sprague-Dawley rats gave the same hydroxylated androstenedione products (data not shown). Prior to analysis, 1 *μ*g of each of the purified hepatic 2B1 and 2B2 samples was run on an SDS−polyacrylamide gel (7.5%) and stained with Coomassie Brilliant Blue, using conditions described previously (*27*).

[3] This 2B2 clone still retains the alterations V322E and M473K present in the $2B2_{FF}$ clone; however, these alterations are not anticipated to influence enzyme activity. Residue 322 is not within an SRS region, and substitution of Met for Lys at residue 473 did not affect the substrate specificity of either 2B1 or a 2B2 variant (*17, 32*).

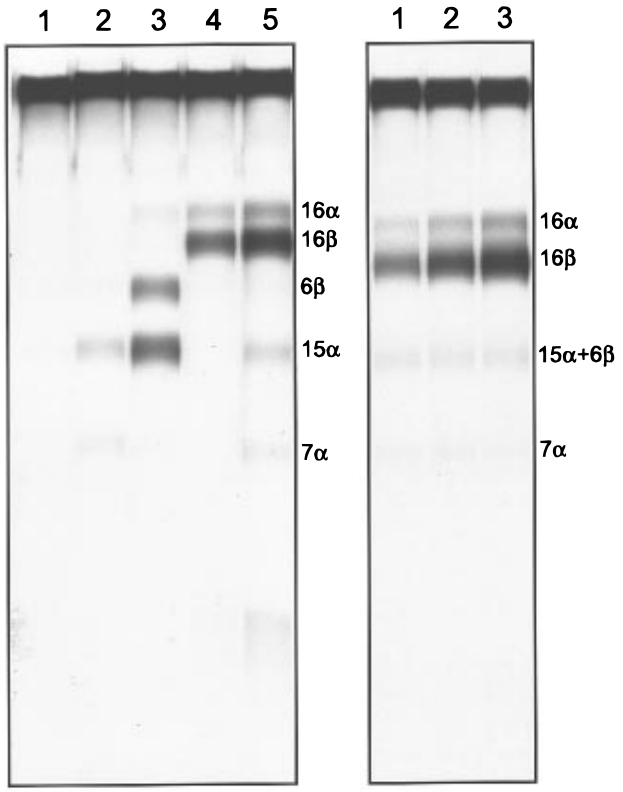

FIGURE 1: Autoradiogram of androstenedione hydroxylation products of *E. coli*-expressed or purified hepatic P450 proteins. Incubations were performed as described in Experimental Procedures. One-third of the reaction mixture was applied to the TLC plate and developed twice in dichloromethane/acetone (4:1, v/v) in the left panel or ethyl acetate/chloroform (2:1, v/v) in the right panel. Migration of standard metabolites is indicated. Enzymes were incubated with [^{14}C] androstenedione for 30 min, with the exception of 2B1 (left panel, lane 4) which was incubated with substrate for 5 min. Left panel: lane 1, control (NADPH omitted from an assay of expressed $2B2_{FF}$); lane 2, $2B2_{FF}$; lane 3, expressed 2B2; lane 4, expressed 2B1; and lane 5, purified hepatic 2B2. Right panel: expressed $2B2_{FF}$ containing 2.5% (lane 1), 5% (lane 2), or 10% (lane 3) expressed 2B1.

As previously ascertained (*27*), the two proteins resolved as single bands of different mobilities, with no evidence of cross-contamination (data not shown). To determine whether $2B2_{FF}$ plus a small percent of 2B1 would give a profile similar to that of purified hepatic 2B2, the two expressed enzymes were mixed in varying amounts. As little as 2.5% of 2B1 present with $2B2_{FF}$ during enzyme reconstitution was sufficient to give predominantly 16α-OH and 16*β*-OH androstenedione (Figure 1, right panel). The ratio of 16-OH products to 7α + 15α + 6*β* products increased with increasing amounts of 2B1, with values of 9.6, 18.5, and 29.9 corresponding to 2.5, 5, and 10% 2B1, respectively. Purified hepatic protein yielded a ratio of 14.3, suggesting that this preparation contained less than 5% of 2B1.

Additional Activities of Expressed 2B2 Enzymes. Other steroidal and nonsteroidal substrates were tested for metabolism by the expressed 2B2 and $2B2_{FF}$ enzymes. Testosterone was a slightly better substrate than androstenedione for both variants, with activities that were nearly 10% of the 2B1 activity (Table 2). $2B2_{FF}$ activity was nearly 10-fold higher for testosterone than for androstenedione, with large increases in 7α- and 16α-hydroxylation. While 15α-hydroxylase activity increased, it accounted for only 20% of the total tesosterone activity, compared with 50% of the androstene-

Table 1: Metabolism of Androstenedione by Wild-Type and Mutant P450 2B Enzymes Expressed in *E. coli* or Purified from Rat Liver[a]

	androstenedione hydroxylase activity[b]						ratio	
P450	7α	15α	6β	16β	16α	total[c]	15α:16-OH	16β:16α
$2B2_{FF}$	0.02 (25)	0.04 (50)	0.01 (12.5)	–	0.01 (12.5)	0.08 (1)	4.0	–
2B2	0.01 (1.5)	0.41 (61)	0.22 (33)	0.01 (1.5)	0.02 (3)	0.67 (5)	13.7	–
2B2 A363V	–	0.02 (7)	0.06 (22)	0.08 (30)	0.11 (41)	0.27 (2)	0.1	0.7
2B2 L367V	0.05 (5)	0.55 (54)	0.34 (34)	0.02 (2)	0.05 (5)	1.01 (8)	7.9	0.4
2B2 A478G	0.08 (2)	2.30 (55)	1.17 (28)	0.34 (8)	0.27 (6)	4.16 (32)	3.8	1.3
2B2 VVG	0.01	0.16 (2)	0.14 (1)	9.25 (89)	0.86 (8)	10.42 (80)	0.02	10.8
2B2 hep. pur.[d]	0.02 (1)	0.07 (4)	0.02 (1)	1.43 (85)	0.14 (8)	1.68 (13)	0.04	10.2
2B1	0.02	0.19 (1)	0.21 (2)	11.39 (87)	1.24 (10)	13.05 (100)	0.02	9.2
2B1 ALA	0.02 (1)	1.21 (75)	0.34 (21)	0.01 (1)	0.03 (2)	1.61 (12)	30.3	–

[a] Results are the means of two or three independent CHAPS-solubilized membrane preparations assayed in duplicate. Activities are expressed in nanomoles of product per minute per nanomole of P450. [b] Numbers in parentheses represent the amount of metabolite as a percentage of the total androstenedione hydroxylase activity. [c] Numbers in parentheses represent the amount of metabolite as a percentage of 2B1 activity. [d] Refers to 2B2 purified from rat liver.

Table 2: Metabolism of Testosterone by Wild-Type and Mutant P450 2B Enzymes Expressed in *E. coli* or Purified from Rat Liver[a]

	testosterone hydroxylase activity[b]						ratio	
P450	7α	15α	6β	16β	16α	total[c]	15α:16-OH	16β:16α
$2B2_{FF}$	0.30 (40)	0.15 (20)	0.02 (3)	–	0.28 (37)	0.75 (5)	0.5	–
2B2	0.01 (1)	0.89 (62)	0.49 (34)	0.02 (1)	0.03 (2)	1.44 (9)	17.8	–
2B2 A363V	0.01 (6)	0.01 (6)	0.03 (17)	0.05 (28)	0.08 (44)	0.18 (1)	0.1	0.6
2B2 L367V	0.07 (3)	1.65 (64)	0.45 (18)	0.15 (6)	0.24 (9)	2.56 (16)	4.2	0.6
2B2 A478G	0.11 (2)	3.40 (56)	1.63 (27)	0.08 (1)	0.83 (14)	6.05 (37)	3.7	0.1
2B2 VVG	0.07 (1)	0.28 (2)	0.20 (2)	6.41 (51)	5.73 (45)	12.69 (77)	0.02	1.1
2B2 hep. pur.[d]	0.20 (10)	0.16 (8)	0.13 (7)	0.62 (32)	0.80 (42)	1.91 (12)	0.1	0.8
2B1	0.01	0.08	0.34 (2)	8.25 (50)	7.76 (47)	16.44 (100)	0.01	1.1

[a] Results are the means of two or three independent CHAPS-solubilized membrane preparations assayed in duplicate. Activities are expressed in nanomoles of product per minute per nanomole of P450. [b] Numbers in parentheses represent the amount of metabolite as a percentage of the total testosterone hydroxylase activity. [c] Numbers in parentheses represent the amount of metabolite as a percentage of 2B1 activity. [d] Refers to 2B2 purified from rat liver.

dione activity. This testosterone hydroxylation profile is in agreement with that of a 2B2 variant similar to $2B2_{FF}$ (*15*). The expressed 2B2 exhibited 15α- and 6β-OH testosterone products at nearly the same percentages as androstenedione (62 and 34%, respectively), with activity increased 2-fold. Again, although $2B2_{FF}$ did have some 16α-hydroxylase activity, the profiles for both $2B2_{FF}$ and expressed 2B2 were in contrast to the 16α- and 16β-hydroxylase activities predominant for 2B1 and the purified hepatic 2B2. The purified hepatic 2B2 yielded higher 7α- and 15α- hydroxylase activities compared with 2B1, again suggesting that it was a mixture of $2B2_{FF}$ and 2B1. There were also some minor unidentified testosterone metabolites for $2B2_{FF}$, expressed 2B2, and purified hepatic 2B2.

In contrast to the difference in activities with androstenedione and testosterone, 2B1 and expressed 2B2 displayed similar hydroxylation rates with progesterone (Table 3). Of the substrates tested, the highest activity of the expressed 2B2 variants was observed with progesterone, whereas 2B1 had a lower activity with this substrate. In fact, 2B1 and expressed 2B2 exhibited similar activities that were about half of that of $2B2_{FF}$. There were also fewer differences in the metabolite profiles with progesterone. For both 2B1 and $2B2_{FF}$, the major metabolite was 16α-OH progesterone (88% of their total activities), whereas for expressed 2B2, the 6β-OH metabolite was predominant (87%) over the 16α-OH metabolite (data not shown). $2B2_{FF}$ also produced 6β-OH progesterone, whereas two minor unidentified metabolites comprised the remaining activity of 2B1.

In addition to steroidal substrates, activities were determined for resorufin derivatives and ethoxycoumarin (Table 3). For 7-benzyloxyresorufin and 7-pentoxyresorufin *O*-dealkylase activities, there was little difference between $2B2_{FF}$ and expressed 2B2, as both enzymes exhibited rates around 1% of those of 2B1. 7-Ethoxycoumarin was a slightly better substrate for $2B2_{FF}$ than for expressed 2B2; however, both enzymes still displayed much lower deethylase activity compared with 2B1 (Table 3).

Table 3: Catalytic Activitites of P450 2B Enzymes Expressed in *E. coli*[a]

	PROG[b]	BROD	PROD	ECOD
$2B2_{FF}$	1.9 (173)[c]	0.10 (1)	0.09 (1)	0.17 (3)
2B2	1.2 (109)	0.11 (1)	0.10 (1)	0.06 (1)
2B1	1.1 (100)	19.9 (100)	7.5 (100)	5.9 (100)

[a] Results are the means of two independent CHAPS-solubilized membrane preparations assayed in duplicate. Activities are expressed in nanomoles of product per minute per nanomole of P450. [b] PROG, progesterone hydroxylase, for 2B2 total activity is from 16α- and 6β-OH progesterone and for 2B1 total activity is from 16α-OH progesterone and several unknown metabolites; BROD, 7-benzyloxyresorufin *O*-debenzylase; PROD, 7-pentoxyresorufin *O*-depentylase; ECOD, 7-ethoxycoumarin *O*-deethylase. [c] Numbers in parentheses are the percentage of the 2B1 enzyme activity.

Mass Spectrometry Analyses of Tryptic Digests and the Partial Peptide Sequence of Purified Hepatic 2B2. Since purified hepatic 2B2 and expressed 2B2 exhibited different steroid metabolite profiles, it was of interest to determine the sequence of the purified hepatic protein. This would establish whether the preparation was predominantly 2B2, as well as which variant was present. Therefore, the purified protein was digested with trypsin and subjected to HPLC−mass spectrometry and tandem mass spectrometry analyses. A tryptic map of the purified hepatic 2B2 is shown in Figure

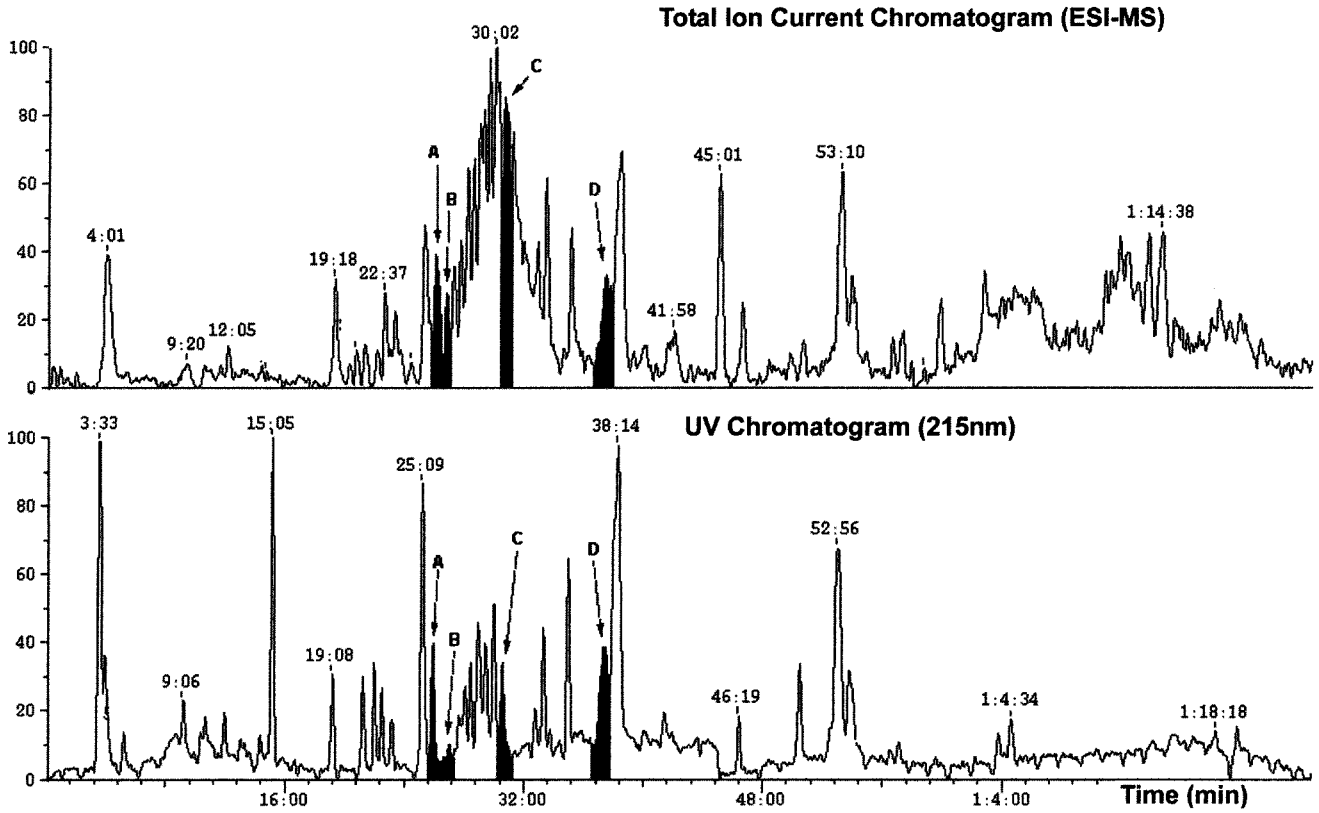

FIGURE 2: UV and electrospray total ion chromatograms of tryptic digests of purified hepatic 2B2. Highlighted peaks represent the peptides that were sequenced by tandem mass spectrometry: peak A, peptide F10 (residues 110−120) with a retention time of 26:01 min, a MW of 1289, and a sequence of EYGVFFANGER; peak B, peptide F35 (residues 317−323) with a retention time of 26:30 min, a MW of 874, and a sequence of YPHVTEK; peak C, peptide F40 (residues 359−370), with a retention time of 30:40 min, a MW of 1308, and a sequence of FADLAPIGLPHR; and peak D, peptide F5 (residues 49−59) with a retention time of 37:13 min, a MW of 1271, and a sequence of GGLLNSFMQFR. Underlined residues are positions altered in $2B2_{FF}$ compared with 2B1.

2. Each of the peptides generated was characterized on the basis of retention time and molecular weight as determined from the singly and doubly protonated molecule signals in the mass spectra. This information was compared to a tryptic map, generated under the same conditions, of purified hepatic 2B1. Peptides were recovered and identified, accounting for 85% of purified 2B2 and 91% of purified 2B1. Peptides were not recovered for residues 62−85, 159−187, and 205−225 of 2B2; however, as these regions do not differ between 2B1 and known 2B2 variants, additional digested protein was not analyzed. The masses of several peptides differed between the purified hepatic 2B1 and 2B2 proteins, in all cases correlating with known differences in DNA sequence, establishing the fact that the purified hepatic preparation did represent 2B2. The data were consistent with identity between the purified hepatic 2B2 and the $2B2_{FF}$ variant. To further verify this, the amino acid sequence of four peptides encompassing residues 49−59, 110−120, 317−323, and 359−370 was determined by CID tandem mass spectrometry. Thus, the protonated molecule of each peptide was subjected to −30 eV of energy, causing dissociation of one or more amide bonds. The resulting spectra contained signals for ion series showing both the N-terminal and C-terminal sequence (*33*−*35*). Analysis of the resulting spectra confirmed the sequence of these peptides, including the presence of Phe58 and Phe114 (data not shown).

Use of Computer Modeling To Map Residues Altered in 2B2 Variants. With the availability of the 2B1 model (*19*), it was of interest to derive a model of $2B2_{FF}$ that would predict residues within the active site that differ between the two enzymes. The $2B2_{FF}$ model was derived from that of 2B1 by replacing the 14 differing amino acids. The side chains of the residues that are altered in 2B2 variants are displayed (Figure 3). The alterations in $2B2_{FF}$ compared with 2B1 are shown in yellow, whereas positions that have been found to be altered in other 2B2 variants are shown in pink. While little is known about their expression or activity, these variants are included to depict the range of substituted positions in 2B2 enzymes. The residues that differ between 2B2 and 2B1 are within the active site (including residues 114, 363, 367, and 478) and scattered over much of the lower two-thirds of the enzyme model as displayed in Figure 3.

Analysis of the Enzyme Activity of Mutant 2B2 Enzymes. On the basis of the $2B2_{FF}$ model and studies of 2B1, we chose to substitute the active site residues 363, 367, and 478 of 2B2 with those present in 2B1. It was of interest to determine how these alterations would influence the metabolite profile of expressed 2B2, and whether the 16-OH metabolites characteristic of 2B1 would be produced. Alteration of each of these residues had an influence on the substrate specificity for steroidal substrates; however, no single substitution conferred a 2B1-like profile, and the extent of the changes in metabolites and activities varied. Comparison of the androstenedione hydroxylase profiles in Figure 4 demonstrates that the 2B2 A363V mutant produced different metabolites than expressed 2B2 (see also Tables 1 and 2). The substitution of valine at position 363 resulted in a shift from 15α- and 6β-hydroxylation (95% of the expressed 2B2 activity) to 16α- and 16β-hydroxylation (72% of the 2B2 A363V activity) for either androstenedione or

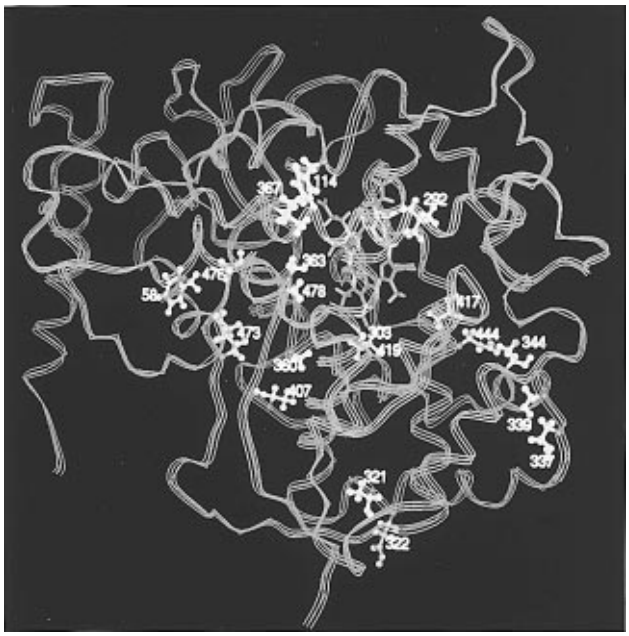

FIGURE 3: Ribbon representation of the P450 $2B2_{FF}$ model showing the location of amino acids altered in 2B2 variants. Heme is shown in red; residue alterations of $2B2_{FF}$ are in yellow, and the positions of residues that are altered in the first reported 2B2 or in other 2B2 variants are shown in pink. Additional 2B2 variants include L292P/G476D (*36*), N444K (*37*), and G438D/G476D (*38*).

testosterone. These two metabolites were present in close to equal amounts, with a 16β:16α ratio of 0.7, much below the ratio of 9.2 observed for 2B1 with androstenedione. The 2B2 A363V mutant also exhibited lower activity compared with expressed 2B2, with only 40% (androstenedione) or 13% (testosterone) of the respective activities of expressed 2B2.

Androgen hydroxylation profiles of the two mutants 2B2 L367V and 2B2 A478G were more similar to that of expressed 2B2, with over 80% of their activity directed toward 15α- and 6β-hydroxylation (Figure 4 and Tables 1 and 2). The 2B2 L367V mutant had a minor reduction in the percent of activity directed toward 15α-OH and 6β-OH products, and a slight increase in 16-OH metabolites. In addition, activity was increased by less than 2-fold compared with expressed 2B2. The 2B2 A478G mutant produced all five metabolites (7α-OH, 15α-OH, 6β-OH, 16α-OH, and 16β-OH) from both steroids, with a 4- or 6-fold increase in activity for testosterone and androstenedione, respectively. Although there were substantial increases in the amounts of the 16α-OH and 16β-OH metabolites, these accounted for only 15% of the total activity of 2B2 A478G with either steroid. A larger increase in 16β-OH activity was observed when the 2B2 L367V alteration was combined with either A363V or A478G (data not shown).

Once all three alterations at residues 363, 367, and 478 were combined in the triple mutant 2B2 VVG, a 2B1-like profile was observed with 16β:16α ratios similar to those obtained for 2B1. For androstenedione, the 16β:16α ratio was 10.8, compared with 9.2 observed for 2B1. For testosterone, the 16β:16α ratio was 1.1 for both 2B2 VVG and 2B1. The total activity of the 2B2 triple mutant was 80% of that of 2B1. The reciprocal mutant, 2B1 ALA, was constructed previously and shown in COS cells to yield metabolites different from those of 2B1 (*17*). 2B1 ALA expressed in *E. coli* exhibited an androstenedione hydroxylation pattern similar to that of expressed 2B2, with 15α- and 6β-hydroxylase activities accounting for 96% of the total activity (Table 1). The total activity and the 15α:16-OH ratio of 2B1 ALA were somewhat higher than those of expressed 2B2.

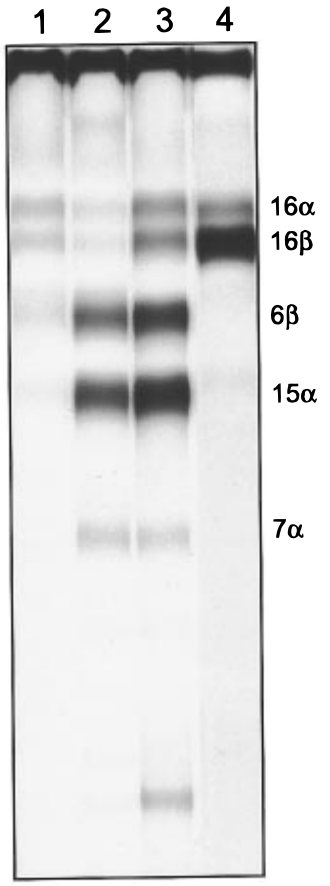

FIGURE 4: Autoradiogram of androstenedione hydroxylation products of *E. coli*-expressed 2B2 mutants. Incubations were performed as described in Experimental Procedures. One-third of the reaction mixture was applied to the TLC plate and developed twice in dichloromethane/acetone (4:1 v/v). Enzymes were incubated with the substrate for the indicated times: lane 1, 2B2 A363V (30 min); lane 2, 2B2 L367V (30 min); lane 3, 2B2 A478G (20 min); and lane 4, 2B2 VVG (10 min).

Inactivation of Expressed 2B2 Variants. Mechanism-based inactivators are valuable probes of functional differences between closely related P450 enzymes. Inactivation studies of the expressed 2B2 enzymes were performed to address the apparent discrepancies in previous studies. A 2B1 mutant with an alanine at residue 478 is refractory to inactivation by the chloramphenicol analog *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide (*13*, *16*). However, purified hepatic 2B2, which also has an alanine at this position as confirmed by mass spectrometry, was reported to be susceptible to inactivation by this compound (*26*). Thus, previous inactivation studies of purified hepatic 2B2 may have been skewed by the presence of 2B1. Expressed 2B2 exhibited diminished inactivation by chloramphenicol with a rate constant of inactivation, k_i, of 0.08 min^{-1} with 250 μM chloramphenicol, whereas $2B2_{FF}$ was refractory (Table 4). Both expressed 2B2 and $2B2_{FF}$ enzymes retained a high percent of activity at zero preincubation time compared with 2B1, especially at the lower concentration of chloramphenicol, which indicates minimal reversible inhibition and suggests impaired binding of chloramphenicol (*18*). Neither

Table 4: Rate Constants for Inactivation[a]

	chloramphenicol		*N*-(2-*p*-nitrophenethyl)-chlorofluoroacetamide
P450	50 μM	250 μM	250 μM
$2B2_{FF}$	0.01 (83)[b]	0.01 (61)	0 (83)
2B2	0.02 (94)	0.08 (66)	0 (45)
2B1	0.14[c] (42)	0.14 (15)	0.17 (42)

[a] k_i values (min^{-1}) are the means of two independent experiments with the indicated inhibitor concentration, conducted as described in Experimental Procedures. Control values (no inhibitor) were all ≤ 0.02 min^{-1}. [b] Numbers in parentheses represent the percent activity remaining at the zero time point, as determined by linear regression analysis.[c] The values for P450 2B1 are similar to those obtained previously: 0.16 min^{-1} for chloramphenicol and 0.13 min^{-1} for the analog (*13, 18*).

$2B2_{FF}$ nor expressed 2B2 was inactivated by *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide, although the competitive inhibition of expressed 2B2 was comparable to that of 2B1.

DISCUSSION

We have shown conclusively that the steroid hydroxylation profiles of two heterologously expressed P450 2B2 variants are different from those of purified hepatic 2B2 preparations. One clone, designated $2B2_{FF}$, was obtained from a cDNA library from phenobarbital-induced Lewis rats and is similar to previously isolated variants containing phenylalanine residues at positions 58 and 114. The second clone was derived from this cDNA clone by restoring the Leu and Ile residues, respectively, at these positions and is designated 2B2. On the basis of numerous reports in the literature (*5*−*7*, *15*), we expected that the expressed 2B2 enzymes would yield a 2B1-like androgen metabolite profile, characterized by the specific production of 16-OH metabolites. However, the expressed $2B2_{FF}$ and 2B2 enzymes exhibited only low androstenedione 15α- and 7α-hydroxylase or 15α- and 6β-hydroxylase activities, respectively. Similar metabolites were obtained for testosterone, with $2B2_{FF}$ also producing low amounts of the 16α-OH metabolite. Thus, the previously established activities of 2B2 based on purified hepatic protein were brought into question.

The absence of 16β- and 16α-OH metabolites in the androstenedione hydroxylation profiles of either expressed 2B2 or $2B2_{FF}$ suggested that the purified hepatic 2B2 protein preparation, which produced these metabolites, might contain some of the higher-activity 2B1 enzyme. In fact, the profile of purified hepatic 2B2 could be reconstructed when <5% of expressed 2B1 was included with $2B2_{FF}$ prior to enzyme reconstitution in an androstenedione hydroxylation assay. Given the large differences between the hydroxylation rates of expressed 2B1 and 2B2, it is easy to understand how the presence of <5% of 2B1 would result in a major contribution to the metabolism of androstenedione. Substrates that are metabolized more equally by these two enzymes, or for which 2B2 has higher activity, would have a smaller 2B1 contribution and less similarity between their profiles. Indeed, differences occur in the ratios of the metabolites formed by either purified 2B2 or purified 2B1 with the substrates 7,12-dimethylbenz[*a*]anthracene (*39*, *40*) or arachidonic acid (*41*, *42*). The putative 2B1 present in purified hepatic 2B2 preparations could be either a 2B1 variant which would be resolved by SDS−PAGE if present in sufficient amounts or a 2B1 variant that comigrates with 2B2. In either case, the low amount of 2B1 present is not detectable by SDS−PAGE or the analytical methods used to assess P450 enzymes, including our mass spectrometry analysis. Two previous studies have noted differences between the ability of purified hepatic 2B2 and an expressed 2B2 variant to metabolize testosterone or 7,12-dimethylbenz[*a*]anthracene (*15*, *39*). However, the significance of these findings was unclear since the sequence of the 2B2 variant present in the purified hepatic 2B2 was not determined. Crucial to the current study was the determination by peptide sequencing that the purified hepatic 2B2 was the same as the $2B2_{FF}$ variant.

Based on a homology model of 2B2, and with the aid of previous studies of 2B1, 2B2 mutants were constructed with substitutions in active site residues 363, 367, and 478. Alteration of these three residues to those present in 2B1 was required to convert expressed 2B2 into a 2B1-like enzyme with high 16β- and 16α-hydroxylase activity. As residues 363, 367, and 478 are conserved among all 2B2 sequences, this is further verification that the substrate specificity of 2B2 enzymes is not likely to be the same as that of 2B1 enzymes. Previous studies of 2B1 mutants with 2B1 → 2B2 substitutions were not fully understood as some of these mutants exhibited steroid hydroxylation profiles not expected for either 2B1 or 2B2. As these activities can now be attributed to expressed 2B2, the role of active site residues 363, 367, and 478 in the interconversion of 2B1 and 2B2 specificities can be assessed. Residue 363 stands out as a major determinant of substrate specificity, with the A363V substitution converting 2B2 from a 15α- and 6β-hydroxylase into a lower-activity 16α- and 16β-hydroxylase. The corresponding alteration in 2B1, V363A, resulted in a loss of 16β-hydroxylase activity and a gain of 15α-hydroxylase activity (*17*, *18*). In contrast, alterations of residues 367 and 478 primarily affected 16β-hydroxylation rates, which were increased in the case of 2B2 → 2B1 substitutions and decreased in the case of 2B1 → 2B2 alterations (*13*, *17*). In addition, the active site residue 114 was also found to influence substrate specificity, as indicated by the different metabolite profiles of $2B2_{FF}$ and expressed 2B2.

The results using *E. coli*-expressed enzymes have important implications for the use of specific inactivators and marker activities in distinguishing between P450 isoforms within microsomes. Because the expressed 2B2 enzymes lack androstenedione 16α- and 16β-hydroxylase and testosterone 16β-hydroxylase activities, which were previously thought to reflect a contribution of both 2B1 and 2B2 (*5*−*7*), these activities can now be considered markers for 2B1 in microsomes. Assessing 2B2 activity in microsomes will continue to be difficult, as the expressed 2B2 variants exhibited low activity toward most of the substrates tested and did not exhibit a unique metabolite for progesterone. The 2B1 inhibitor *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide was previously thought to inactivate 2B2, on the basis of work with the purified hepatic enzyme (*26*), but it has now been shown to be selective for 2B1. The lack of 2B2 inactivation is consistent with previous results showing that an alanine at position 478 makes 2B1 refractory to inactivation by this compound (*12*, *13*). It is now of interest to retest other inhibitors and to search for selective inactivators of each of the 2B2 variants. The 2B2 enzyme model will be useful in predicting derivatives that may inactivate these enzymes.

The elucidation of the activities of the expressed 2B2 enzymes increases the correlations that can be made between the rat and rabbit members of the 2B subfamily. Previously, it was thought that the androstenedione 15α-hydroxylase activity of the rabbit 2B5 enzyme was unique among the 2B enzymes (*11*, *45*). However, expressed 2B2 variants also exhibited this activity. Rat 2B1 and rabbit 2B4 are also similar to each other in substrate specificities and are highly selective androstenedione 16*β*-hydroxylases. In addition, the expression of 2B2 and 2B5 is hepatic, and even lacking in certain animals, whereas 2B1 and 2B4 are expressed in both hepatic and extrahepatic tissues such as the lung (*43*−*46*). The possible implications of these similarities and differences among the 2B subfamily in terms of the evolution and selection of P450 enzymes are intriguing.

While several different 2B2 sequences have been described in the literature, the L58F/I114F alteration is predominant among 2B2 variants. Four independent clones that contain this alteration have been isolated from Sprague-Dawley rats, along with either lysine or methionine at residue 473 (*15*, *30*, *31*, *47*). Another 2B2 L58F/I114F variant containing an additional seven peptides after residue 275 (due to alternate splicing) has been found in phenobarbital- and Aroclor 1254-induced Sprague-Dawley rats (*32*). Our isolation of $2B2_{FF}$ from a cDNA library from phenobarbital-induced Lewis rats and identification of this variant as the hepatic 2B2 purified from phenobarbital-induced Sprague-Dawley rats suggest that it is the major phenobarbital-inducible 2B2 isozyme in these animals. However, a different 2B2 variant has been purified from Long-Evans rats. The protein sequence of this hepatic 2B2 reveals that it is similar to the first published 2B2 nucleotide sequence, and not the $2B2_{FF}$ variant (*48*). Two different 2B2 proteins, designated PB5 and PB8, have been identified on the basis of the separation of microsomal proteins by two-dimensional gel electrophoresis (*49*). Although Long-Evans and Lewis rats express different 2B2 variants on the basis of sequence analysis, they have both been typed as expressing PB5 (*50*, *51*). This suggests that PB5 represents at least two variants, $2B2_{FF}$ and 2B2. Although little is known about their expression, other 2B2 variants with alterations in residues 292, 322, 444, 476, or 473 have also been cloned (*36*−*38*). These residues are not predicted by the computer model to be close to the active site, suggesting that steroid substrate specificities for these variants may be similar to those of expressed 2B2 or $2B2_{FF}$.

The finding that a minor amount of 2B1 can significantly affect the apparent activities of purified hepatic 2B2 gives a note of caution for the use of purified protein preparations, especially when enzyme activities are low and the chance of copurification of a higher-activity enzyme exists. Depending upon the substrate, previous work using purified hepatic 2B2 preparations may be biased. Heterologously expressed 2B2 can be used to confirm the metabolites assigned to purified hepatic 2B2 preparations. In addition, the expressed 2B2 enzymes have provided further support for the key residues involved in the substrate specificity of 2B enzymes. The 2B subfamily will continue to offer insight into the function and evolution of P450 enzymes as additional members are characterized (*47*, *52*).

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REFERENCES

1. Nelson, D. R. (1995) in *Cytochrome P450: Structure, Mechansism, and Biochemistry*, (Ortiz de Montellano, P. R., Ed.) 2nd ed., pp 575−606, Plenum Press, New York.
2. Ryan, D. E., and Levin, W. (1990) *Pharmacol. Ther. 45*, 153−239.
3. Fujii-Kuriyama, Y., Mizukami, Y., Kawagiri, K., Sagawa, K., and Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793−2797.
4. Mizukami, Y., Sobawa, K., Suwa, Y., Muramatsu, J., and Fujii-Kuriyama, Y. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3958−3962.
5. Waxman, D. J., Ko, A., and Walsh, C. (1983) *J. Biol. Chem. 258*, 11937−11947.
6. Ryan, D. E., Thomas, P. E., and Levin, W. (1982) *Arch. Biochem. Biophys. 216*, 272−288.
7. Wood, A. W., Ryan, D. E., Thomas, P. E., and Levin, W. (1983) *J. Biol. Chem. 258*, 8839−8847.
8. Swinney, D. C., Ryan, D. E., Thomas, P. E., and Levin, W. (1987) *Biochemistry 26*, 7073−7083.
9. Gasser, R., Negishi, M., and Philpot, R. M. (1988) *Mol. Pharmacol. 33*, 22−30.
10. Szklarz, G. D., He, Y. Q., Kedzie, K. M., Halpert, J. R., and Burnett, V. L. (1996) *Arch. Biochem. Biophys. 327*, 308−318.
11. Kedzie, K. M., Philpot, R. M., and Halpert, J. R. (1991) *Arch. Biochem. Biophys. 291*, 176−186.
12. Kedzie, K. M., Balfour, C. A., Escobar, G. Y., Grimm, S. W., He, Y., Pepperl, D. J., Regan, J. W., Stevens, J. C., and Halpert, J. R. (1991) *J. Biol. Chem. 266*, 22515−22521.
13. He, Y., Balfour, C. A., Kedzie, K. M., and Halpert, J. R. (1992) *Biochemistry 31*, 9220−9226.
14. John, G. H., Hasler, J., He, Y., and Halpert, J. R. (1994) *Arch. Biochem. Biophys. 314*, 367−375.
15. Aoyama, T., Korzekwa, K., Nagata, K., Adesnik, M., Reiss, A., Lapenson, D. P., Gillette, J., Gelboin, H. V., Waxman, D. J., and Gonzalez, F. J. (1989) *J. Biol. Chem. 264*, 21327−21333.
16. Halpert, J. R., and He, Y. (1993) *J. Biol. Chem. 268*, 4453−4457.
17. He, Y., Luo, Z., Klekotka, P. A., Burnett, V. L., and Halpert, J. R. (1994) *Biochemistry 33*, 4419−4424.
18. He, Y. Q., He, Y. A., and Halpert, J. R. (1995) *Chem. Res. Toxicol. 8*, 574−579.
19. Szklarz, G. D., He, Y., and Halpert, J. R. (1995) *Biochemistry 34*, 14312−14322.
20. Gotoh, O. (1992) *J. Biol. Chem. 267*, 83−90.
21. He, Y. Q., Szklarz, G. D., and Halpert, J. R. (1996) *Arch. Biochem. Biophys. 335*,152−160.
22. Harlow, G. R., and Halpert, J. R. (1997) *J. Biol. Chem. 272*, 5396−5402.
23. Omura, T., and Sato, R. (1964) *J. Biol. Chem. 239*, 2379−2387.
24. Hasler, J. A., Harlow, G. R., Szklarz, G. D., John, G., Kedzie, K. M., Burnett, V. L., He, Y., Kaminsky, L. S., and Halpert, J. R. (1994) *Mol. Pharmacol. 46*, 338−345.
25. Grimm, S. W., Dyroff, M. C., Philpot, R. M., and Halpert, J. R. (1994) *Mol. Pharmacol. 46*, 1090−1099.
26. Halpert, J., Jaw, J.-Y., Balfour, C., and Kaminsky, L. S. (1990) *Drug Metab. Dispos. 18*, 168−174.
27. Graves, P. E., Kaminsky, L. S., and Halpert, J. R. (1987) *Biochemistry 26*, 3887−3894.
28. Halpert, J., Balfour, C., Miller, N., Morgan, E. T., Dunbar, D., and Kaminsky, L. (1985) *Mol. Pharmacol. 28*, 290−296.
29. Szklarz, G. D., Ornstein, R. L., and Halpert, J. R. (1994) *J. Biomol. Struct. Dyn. 12*, 61−78.

30. Affolter, M., and Anderson, A. (1984) *Biochem. Biophys. Res. Commun. 118*, 655−662.
31. Lacroix, D., Desrochers, M., Lambert, M., and Anderson, A. (1990) *Gene 86*, 201−207.
32. Desrochers, M., Christou, M., Jefcoate, C., Belzil, A., and Anderson, A. (1996) *Biochem. Pharmacol. 52*, 1311−1319.
33. Biemann, K. (1988) *Biomed. Environ. Mass Spectrom. 16*, 99−111.
34. Thibault, P., Pleasance, S., Laycock, M. V., MacKay, R. M., and Boyd, R. K. (1991) *Int. J. Mass Spectrom. Ion Processes 111*, 317−353.
35. Grieve, P. A., Jones, A., and Alewood, P. F. (1993) *J. Chromatogr. 646*, 175−184.
36. Frey, A. B., Waxman, D. J., and Kreibich, G. (1985) *J. Biol. Chem. 260*, 15253−15265.
37. Phillips, I. R., Shephard, E. A., Ashworth, A., and Rabin, B. R. (1983) *Gene 24*, 41−52.
38. Kumar, A., Raphael, C., and Adesnik, M. (1983) *J. Biol. Chem. 258*, 11280−11284.
39. Christou, M., Mitchell, M. J., Jovanovich, M. C., Wilson, N. M., and Jefcoate, C. R. (1989) *Arch. Biochem. Biophys. 270*, 162−172.
40. Christou, M., Mitchell, M. J., Aoyama, T., Gelboin, H. V., Gonzalez, F. J., and Jefcoate, C. R. (1992) *Biochemistry 31*, 2835−2841.
41. Capdevila, J. H., Karara, A., Waxman, D. J., Martin, M. V., Falck, J. R., and Guenguerich, F. P. (1990) *J. Biol. Chem. 265*, 10865−10871.
42. Laethem, R. M., Halpert, J. R., and Koop, D. R. (1994) *Biochim. Biophys. Acta 1206*, 42−48.
43. Omiecinski, C. J. (1986) *Nucleic Acids Res. 14*, 1525−1539.
44. Omiecinski, C. J., Ramsden, R., Rampersaud, A., and Walz, F. G., Jr. (1992) *Mol. Pharmacol. 42*, 249−256.
45. Ryan, R., Grimm, S. W., Kedzie, K. M., Halpert, J. R., and Philpot, R. M. (1993) *Arch. Biochem. Biophys. 304*, 454−463.
46. Traber, P. G., Wang, W., McDonnell, M., and Gumucio, J. J. (1990) *Mol. Pharmacol. 37*, 810−819.
47. Atchison, M., and Adesnik, M. (1983) *J. Biol. Chem. 258*, 11285−11295.
48. Yuan, P.-M., Ryan, D. E., Levin, W., and Shively, J. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1169−1173.
49. Rampersaud, A., and Walz, F. G., Jr. (1987) *Biochem. Genet. 25*, 527−534.
50. Rampersaud, A., and Walz, F. G., Jr. (1983) *Proc. Natl. Acad. Sci. U. S. A. 80*, 6542−6546.
51. Rampersaud, A., and Walz, F. G., Jr. (1987) *J. Biol. Chem. 262*, 5649−5653.
52. Giachelli, C. M., Lin-Jones, J., and Omiecinski, C. J. (1989) *J. Biol. Chem. 264*, 7046−7053.

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