Selective Suppression of the Catalytic Activity of cDNA-Expressed Cytochrome P4502B1 toward Polycyclic Hydrocarbons in the Microsomal Membrane: Modification of This Effect by Specific Amino Acid Substitutions[†,‡]

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ABSTRACT: Human hepatoma HEPG2 cells were infected with recombinant vaccinia virus vectors containing cDNAs encoding both known and variant rat cytochromes P450 (CYP). CYP2B1 and CYP2B2 cytochromes were equally well expressed (110–140 pmol/mg of microsomal protein) and catalyzed metabolism of 7,12-dimethylbenz[*a*]anthracene (DMBA). Their regioselectivity for DMBA metabolism paralleled that of the respective purified rat liver enzymes and reproduced previously reported regioselective differences between CYP2B1 and CYP2B2 [Wilson et al. (1984) *Carcinogenesis 5*, 1475–1483]. CYP2A1 and CYP2A2 expressed in HEPG2 microsomes exhibited nearly equal DMBA-metabolizing activities that closely matched that of purified CYP2A1. Although purified rat liver CYP2B1 was 3 times more active than purified rat liver CYP2B2, the expressed recombinant microsomal CYP2B1 (rCYP2B1) was 20 times less active than rCYP2B2, where activity matched that of the purified cytochrome. Microsomal suppression of rCYP2B1 catalytic activity was also observed for benzo[*a*]pyrene. Specific amino acid substitutions at equivalent positions of the completely homologous NH_2-terminal halves of rCYP2B1 and rCYP2B2 changed this suppression effect. Thus, a $L^{58} \rightarrow F$, $I^{114} \rightarrow F$ double mutant exhibited 3 times the normal activity for rCYP2B1 while remaining inhibitory for rCYP2B2. The single substitutions produced very different effects. The $L^{58} \rightarrow F$ substitution prevented expression of rCYP2B1, while the $I^{114} \rightarrow F$ substitution was inhibitory for both rCYP2B1 and rCYP2B2 (40 and 70%). A single $E^{282} \rightarrow V$ mutation produced a stimulation of rCYP2B1 activity comparable to that of the $L^{58} \rightarrow F$, $I^{114} \rightarrow F$ double substitution. Since this double mutation decreases testosterone metabolism by rCYP2B1 [Aoyama et al. (1989) *J. Biol. Chem. 264*, 21327–21333], the selective effects on DMBA metabolism by rCYP2B1 mutants may result from relief of the membrane restriction that affects this bulky lipophilic substrate.

Cytochrome P450 (CYP)[1] constitutes a superfamily of proteins, many of which catalyze a broad variety of monooxygenase reactions (Gonzalez, 1988). Most forms of P450, present in the microsomal membranes, function in the metabolism of xenobiotics and typically exhibit a broad range of substrate specificities and regioselectivities for individual substrates (Waxman, 1988; Kaminsky & Guengerich, 1985; Conney, 1982). Reconstitution of purified microsomal P450 cytochromes usually displayed optimal activity with stoichiometric amounts of NADPH-P450 oxidoreductase (Kaminsky & Guengerich, 1985) and complex stimulation by phospholipids and cytochrome b_5 (Ingelman-Sundberg et al., 1981; Waxman & Walsh, 1983).

The P450 superfamily has been divided into a number of families and subfamilies based on the extent of sequence relatedness of the purified proteins (Gonzalez, 1988; Nebert et al., 1989). The two major members of the CYP2B subfamily in rats, CYP2B1 and CYP2B2 (P450b and P450e), differ by only 13 amino acids (Suwa et al., 1985). While CYP2B1 is typically at least 3-fold more active for most substrates (Guengerich et al., 1982; Wolf et al., 1988), the two forms generally exhibit similar regioselectivity (Waxman et al., 1983). In spite of these similarities, expression of CYP2B genes is tissue-specific in untreated rats (Omiecinski, 1986; Christou et al., 1987). For example, CYP2B2, but not CYP2B1, is expressed constitutively in rat liver, while the reverse expression is seen in lung. In rats, the major site of expression of CYP2B proteins is phenobarbital-induced liver, where CYP2B1 and CYP2B2 contribute over 10% of total microsomal protein and exist at a respective ratio of 2:1 (Christou et al., 1987).

Microsomes typically contain several forms of P450 that may contribute to the metabolism of a single substrate. The contributions of individual forms have been assessed from the formation of characteristic metabolites and from the selective inhibitory effects of specific antibodies (Waxman, 1988; Reik et al., 1985; Christou et al., 1989). The membrane disposition of P450 may substantially affect microsomal activities through effects of phospholipids on the conformation of the P450 and the interactions with cytochrome b_5 and NADPH-P450 oxidoreductase. For example, testosterone 6β-hydroxylation by purified CYP2C6 (P450 PB-1) is highly stimulated by phosphatidylserine and by cytochrome b_5 (Oeda et al., 1985). We have recently distinguished the contributions of CYP2B1 and CYP2B2 in microsomal metabolism on the basis of differences in 7- and 12-methylhydroxylation of DMBA (Wilson

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[‡] This paper is dedicated to Neil Wilson, who started this study but whose promising career has been prematurely terminated by illness.

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[1] Abbreviations: CYP, cytochrome P450; DMBA, 7,12-dimethylbenz[*a*]anthracene; BP, benzo[*a*]pyrene; r, recombinant; AHS, aminohexyl-Sepharose.

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et al., 1984). Surprisingly, this resolution of the activities showed that in microsomes from F344 rats CYP2B1 was far less active than the purified cytochrome, while microsomal CYP2B2 functioned in accordance with the expected activity of the pure enzyme (Christou et al., 1989). Inbred M520/N rats that do not express CYP2B2 even more clearly demonstrated this membrane suppression of DMBA metabolism at CYP2B1 (Christou et al., 1989).

Expression of P450 cDNAs in a variety of host cells has resulted in the production of the corresponding active cytochrome (Battula et al., 1987; Zuber et al., 1988; Faletto et al., 1988). An effective means of cDNA expression involves the use of vaccinia virus constructs in which P450 cDNAs are incorporated into the viral genome under control of viral-derived transcriptional regulatory sequences (Faletto et al., 1988). Expression of functional mouse CYP1A1 and CYP1A2 (P_1 and P_3) into murine and human cells was first described (Faletto et al., 1988), and more recently the same approach has also been applied to expression of rat CYP2B1, CYP2A1, and CYP2A2 in HEPG2 cells (Faletto et al., 1988; Aoyama et al., 1990). HEPG2 cells have been chosen because of the negligible base-line PAH metabolism in wild-type cells.

In the present report, we show that previously reported activity and regioselective differences in the metabolism of DMBA by purified CYP2B1 and CYP2B2 (Wilson et al., 1984) are also observed with the cDNA-expressed proteins and that the restriction of CYP2B1-catalyzed microsomal DMBA metabolism also occurs in microsomes from HEPG2 cells following expression therein of CYP2B1 cDNA. Partial purification of cDNA-expressed CYP2B1 and CYP2B2 forms has been used to estimate the contribution of the microsomal membrane to this selective restriction.

The effects of three distinct amino acid substitutions at equivalent positions in cDNAs coding for CYP2B1 and CYP2B2 have been used here to further define the structural requirements for suppression of DMBA metabolism. We show that the effects of membranes on these recombinant P450 cytochromes are similar to the analogous suppression processes in liver microsomes and are partially reversed by two amino acid substitutions.

Materials and Methods

Recombinant Viruses. Construction of vaccinia viruses containing the CYP2B1 cDNAs and the various chimeric derivatives have been described (Aoyama et al., 1989). The sequence of CYP2B2 is identical to that determined by Atchison and Adesnik (1983). The cDNAs were inserted into vaccinia virus, as described by Chakrabarti et al. (1985), using a vector pSC11 and wild-type virus strain WR (Chakrabarti et al., 1985). The various recombinant viruses were used to infect human HEPG2 cells.

Subfractionation and Purification Methods. Isolation of microsomes and aminohexyl-agarose chromatography of P450 cytochromes from infected HEPG2 cells was carried out essentially as described recently for C3H10T-1/2 cells (Pottenger & Jefcoate, 1990). CYP2B1 and CYP2B2 were purified using rat liver microsomes isolated from phenobarbital-treated F344 rats according to published procedures (Funae & Imaoka, 1985). Pure rat liver CYP2A1 was generously donated by Mr. W. Levin (Hoffman LaRoche).

Quantitation of Cytochromes P450. The total cytochrome P450 content of microsomes was determined from the CO/dithionite difference spectra (Omura & Sato, 1964). The apoprotein levels of CYP2B isozymes were determined from Western blots in comparison with purified P450 CYP2B1 and CYP2B2 standards using an antibody to rat CYP2B1, as previously described (Guengerich et al., 1982; Christou et al., 1987).

Table I: Cytochrome P450 Content of Preparations

fraction	specific content (pmol/mg of protein)
recombinant P450/HEPG2 cells	
normal CYP2B1	
microsomes	140
AHS eluate	600
normal CYP2B2	
microsomes	110
AHS eluate	1000
PB-induced rat liver microsomes	
microsomes	1690 (800[a] + 300[b])
AHS eluate	3200 (2100[a] + 900[b])

[a] Content of CYP2B1. [b] Content of CYP2B2.

Quantitation of Polycyclic Hydrocarbon Metabolism. Microsomal metabolism of DMBA and benzo[*a*]pyrene (BP) and analysis of the products by HPLC chromatography were carried out as described elsewhere (Christou et al., 1989).

Materials. Vaccinia virus strain WR, pSC11, and human TK-143 cells used in the construction of the vaccinia virus recombinants were obtained from Dr. Bernard Moss at the National Institutes of Health. HEPG2 cells (ATCC HB 8065) and VC-1 (ATCC CCL 70) cells were from the American Type Culture Collection, Rockville, MD.

Results

Quantitation of Recombinant Cytochromes P450. Comparison between the levels of immunoquantifiable vaccinia virus-exposed recombinant CYP2B1 (rCYP2B1) and CYP2B2 (rCYP2B2) apoproteins and total spectrally detectable cytochrome P450 levels (Table I) demonstrated that the synthesis of total rCYP2B proteins compared closely to the levels of the complete spectrally active cytochromes and that these levels were the same for each construct (110–140 pmol/mg of protein). Very low levels of spectrally detectable P450s were observed in the uninfected HEPG2 cells, but no immunoreactivity was exhibited with anti-CYP2B1 (data not shown).

Partial purification of rCYP2B1 and rCYP2B2 by detergent solubilization and aminohexyl-Sepharose (AHS) chromatography produced, respectively, 4- and 9-fold enrichment of both cDNA-expressed P450s (Table I). These partially purified preparations were predominantly lipid-free and were used for reconstitution experiments in parallel with purified rat liver CYP2B1 and CYP2B2.

DMBA Metabolism by rCYP2B1 and rCYP2B2. Activities and regioselectivity for metabolism of DMBA (Figure 2) by microsomal and partially purified rCYP2B1 and rCYP2B2 from HEPG2 cells were determined and compared to those observed with liver microsomes from PB-induced male F344 rats and by partially or fully purified liver CYP2B1 and CYP2B2 preparations (Table II). All partially or fully purified P450s were reconstituted with oxidoreductase and epoxide hydratase, as previously described (Wilson et al., 1984). Microsomes containing either rCYP2B1 or rCYP2B2 formed 5,6- and 8,9-dihydrodiols and both 7- and 12-hydroxymethyl metabolites of DMBA. These activities were completely inhibited by an antibody to rat liver CYP2B1. Addition of exogenous purified epoxide hydratase to HEPG2 microsomes in vitro did not enhance dihydrodiol formation (data not shown). Phenols that can form by rearrangement of epoxide intermediates were undetectable. Both observations indicate a sufficiency of constitutively expressed epoxide hydratase in the HEPG2 cells for complete hydration of epoxide intermediates.

Table II: Effects of Partial Purification of Recombinant Microsomal CYP2B1 and CYP2B2 on DMBA Metabolism

	DMBA metabolites (pmol nmol^{-1} min^{-1})					
	dihydrodiols			hydroxymethyl		
enzyme source	5,6-	8,9-	3,4-	7-OH	12-OH	total
rCYP2B1 in HEPG2						
microsomes	20	5	1	26	17	68
AHS eluate[a]	100	24	1	125	85	335
rCYP2B2 in HEPG2						
microsomes	35	15	1	230	1000	1280
AHS eluate[a]	50	22	1	150	1060	1282
PB-RLMs						
microsomes	250	370	340	200	530	1690
AHS eluate[b]	80	45	15	90	110	325[c]
pure CYP2B1	780	150	10	565	270	1735
pure CYP2B2	190	35	10	150	230	605

[a] In all cases, 0.1% Emulgen 911 was substantially decreased by several treatments with Bio-Beads. Sodium cholate (0.4%) was removed by extensive dialysis. [b] This fraction contains predominantly P450CYP2B1 and CYP2B2, as shown in Table I. [c] Bio-Bead treatments of this sample failed to lower Emulgen 911 to below inhibitory levels.

DMBA metabolism by microsomes from HEPG2 cells expressing rCYP2B proteins exhibited similar regioselectivities to metabolism catalyzed by the corresponding purified hepatic CYP2B proteins. For microsomal rCYP2B1 and purified CYP2B1, rates of DMBA 5,6-oxidation were 4 times faster than 8,9-oxidation and similar to 7-methylhydroxylation, while there was a total absence of attack at the 3,4-position. Microsomal rCYP2B2 was 20 times more active than microsomal rCYP2B1 and exhibited a specific activity very similar to that of the purified liver CYP2B2. There were also quantitative differences between metabolism catalyzed by recombinant versus hepatic CYP2B2 (rCYP2B2 produces much less 5,6-dihydrodiol relative to 8,9-dihydrodiol and a much higher proportion of the 12-hydroxymethyl derivative of DMBA). The differences in regioselectivity between rCYP2B1 and rCYPB2 were similar to those previously reported for the purified rat CYP2B1 and CYP2B2 (Wilson et al., 1984); notably, rCYP2B2 exhibited lower 5,6-oxidation relative to 8,9-oxidation (ratio 2 versus 4) and a much higher ratio of 12-methylhydroxylation relative to 7-methylhydroxylation (ratio 4.5 versus 0.6).

Recent studies in our laboratory revealed that DMBA metabolism by microsomal CYP2B1 was greatly suppressed relative to that seen with the respective purified and reconstituted cytochrome, whereas metabolism by microsomal CYP2B2 was similar to that of the purified isozyme (Christou et al., 1989). A similar selective suppressive effect was observed with cDNA-expressed CYP2B forms in HEPG2 cells (Table II). Thus, DMBA metabolism by microsomal rCYP2B1 was 25 times slower than that by purified rat liver CYP2B1 and was 20 times slower than that by microsomal rCYP2B2. Partial purification of rCYP2B1 produced a 5-fold increase in DMBA turnover, while no such increase was seen for rCYP2B2. Thus, the reconstituted rCYP2B1 activity was still 4-fold less than the DMBA metabolism by similarly treated rCYP2B2, even though fully purified hepatic CYP2B1 was 5 times more active than CYP2B2. Similar DMBA-metabolizing activity was observed for an equivalent fraction prepared from phenobarbital-induced rat liver microsomes, and this rate was 5 times slower than for purified CYP2B1. Incomplete removal of detergent from these very small samples is a major cause for these diminished activities. More extensive removal of detergent by hydroxylapatite treatment raised activities for larger scale partially purified liver fractions (data not shown). Interestingly, partially purified rCYP2B2 was less sensitive to the effects of residual Emulgen 911.

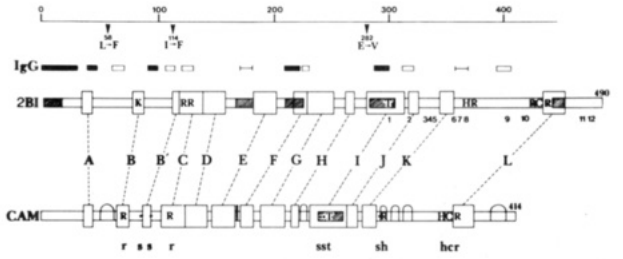

FIGURE 1: Structural similarities between *Pseudomonas putida* $P450_{CAM}$ and CYP2B1. The top scale represents the amino acid number and mutations introduced into CYP2B1. Conserved helical domains are designated by A–L for rat CYP2B1 and $P450_{CAM}$. For $P450_{CAM}$, this analysis is obtained from the X-ray structure (Poulos et al., 1985, 1987), and for CYP2B1, this is designated from published sequence alignments (Edwards et al., 1989). Shaded sections represent very hydrophobic regions determined from hydropathy plots (Edwards et al., 1991). Numbers 1–12 for the CYP2B1 sequence designate sequence differences between rat CYP2B1 and CYP2B2 retained for all cDNAs (Sawa et al., 1985; Atchison & Adesnik, 1983). In the second line, closed rectangles represent regions of CYP2B1 that are bound by IgG raised against these sequences when the protein is solubilized but are protected by membrane insertion (DeLemos-Chiarandini et al., 1987). Regions indicated by open rectangles are bound by peptide-specific IgG in the membrane-bound state, while single lines indicate regions that are protected from such IgG interactions, even in the soluble state. The last line indicates several other conserved features of $P450_{CAM}$ and CYP2B1: c, conserved heme-binding cysteine (C); h, positively-charged amino acids that bind heme carboxyls (K, R, H); r, binds negative domains of reducing proteins (Stayton et al., 1989; Shimuzu et al., 1991); s(·), amino acids contacting the substrate when bound; t, conserved threonine (T) that contacts complexed O_2; open loops, β-sheet structures.

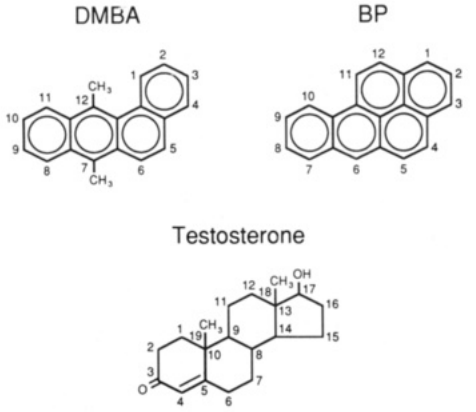

FIGURE 2: Chemical structures of DMBA, BP, and testosterone.

Effects of Specific Amino Acid Substitutions on DMBA Metabolism by rCYP2B1 and rCYP2B2. The sequences of CYP2B1 and CYP2B2 are identical for the first 302 amino acids (Guengerich et al., 1982). Single and multiple substitutions were made in the CYP2B1 and CYP2B2 sequences at positions 58 (Leu → Phe), 114 (Ile → Phe), and 282 (Glu → Val) (Figure 1). Each substitution corresponds to differences in sequence detected in CYP2B2 clones isolated from two rat liver cDNA libraries and, therefore, presumably to natural variants (Aoyama et al., 1989). Although all possible combinations of substitutions were constructed for P450 CYP2B1, the single substitution at position 58 and the double substitution at positions 58 and 282 resulted in unstable CYP2B2 variants (Aoyama et al., 1989). The expression of each of the remaining rCYP2B2 variants in HEPG2 microsomes was similar to the expression of the initial form of the cytochrome (approximately 100 pmol/mg of microsomal protein).

DMBA metabolism from these four rCYP2B1 variants was compared with the products from the initial rCYP2B1 and

Table III: Metabolism of DMBA by Recombinant CYP2B1 and CYP2B2 Variants Expressed in HEPG2 Cells

	DMBA metabolites (pmol nmol^{-1} min^{-1})				
	dihydrodiols		hydroxymethyl		
	5,6-	8,9-	7-OH	12-OH	total
uninfected	nd[a]	nd	nd	2	2
normal CYP2B1	20	5	26	17	68
b_2	13	2	14	9	38
b_{12}	45	15	72	58	190
b_3	44	7	44	33	125
b_{123}	30	10	51	58	149
normal CYP2B2	35	15	230	1000	1280
e_2	28	1	52	290	371
e_{12}	30	1	55	330	416

[a] nd, not detectable.

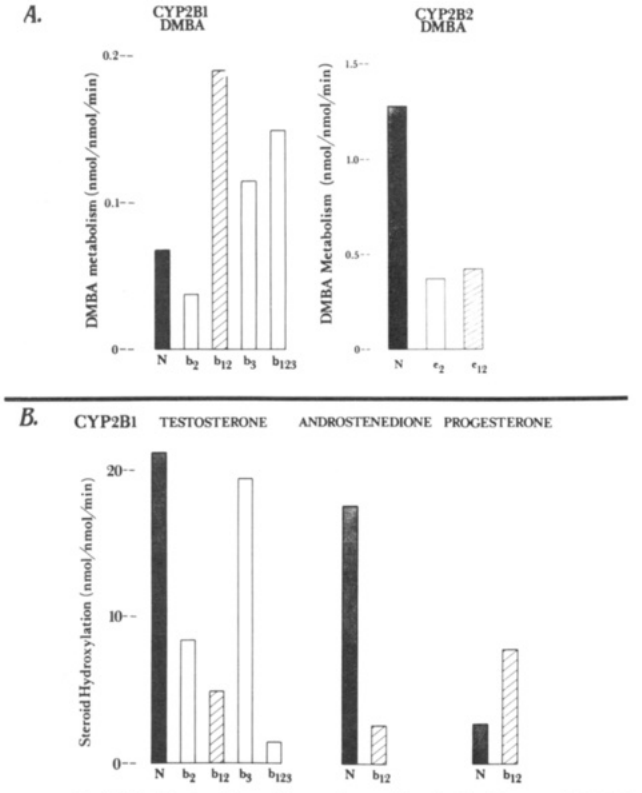

FIGURE 3: (A) Effects of specific amino acid substitutions on DMBA metabolism by CYP2B1 (b-mutants) and CYP2B2 (e-mutants). Subscript numbers designate substitutions: 1, $L^{56} \rightarrow F$, 2, $I^{114} \rightarrow F$, and 3, $E^{282} \rightarrow V$; 12 and 123 indicate multiple substitutions. (B) Effects of the same substitutions on steroid metabolism previously reported by Aoyama et al. (1989).

rCYP2B2 cytochromes (Table III; Figure 3A). Each metabolite profile represents the mean of two separate infection and metabolism experiments with the same vaccinia virus construct. The $I^{114} \rightarrow F$ substitution (b_2) decreased metabolism 2-fold for each product, while the $E^{282} \rightarrow V$ substitution (b_3) increased metabolism by 2-fold for each product. Surprisingly, a double rCYP2B1 mutant containing a $I^{114} \rightarrow F$ substitution, combined with the destabilizing $L^{58} \rightarrow F$ substitution, produced a stable P450 (b_{12}) with 3-fold greater activity than that of the initial rCYP2B1. When all three positions were substituted (b_{123}), the activity remained similarly elevated. None of these substitutions greatly affected the positional selectivity.

The stimulatory effect of this double substitution was not seen with the equivalent rCYP2B2 variants. Substitution at position 114 was again very inhibitory, while the additional substitution at position 58 had no effect on activity. Thus, when rCYP2B1 and rCYP2B2 each have phenylalanine at positions 58 and 114, DMBA metabolism activities converge to within a factor of 2 instead of the 20-fold difference seen for the parental rCYP2B cytochromes.

Table IV: Metabolism of BP by Recombinant CYP2B1 and CYP2B2 Variants Expressed in HEPG2 Cells

	BP metabolites (pmol nmol^{-1} min^{-1})		
microsomal recombinants	4,5-diol	3-phenol	total
normal CYP2B1	nd[a]	2	2
b_2	nd	nd	nd
b_{12}	nd	3	3
b_3	nd	2	2
b_{123}	nd	2	2
normal CYP2B2	136	13	149
e_2	46	1	47
e_{12}	51	1	51
pure liver enzymes			
CYP2B1	215	130	380
CYP2B2	192	17	240

[a] nd, not detectable.

Table V: DMBA Metabolism by Recombinant CYP2A1 and CYP2A2 Expressed in HEPG2 Cells

	DMBA metabolites [pmol (nmol of P450)$^{-1}$ min^{-1}]						
	dihydrodiols			hydroxymethyl			
P450	5,6-	8,9-	3,4-	7-OH	12-OH	phenols	total[a]
rCYP2A1	80	12	4	60	240	8	404
rCYP2A2	50	20	5	60	230	6	371
pure CYP2A1	98	10	2	70	326	4	508

[a] Values calculated on the basis of a specific content of 60 and 25 pmol/mg of protein for rCYP2A1 and rCYP2A2, respectively.

BP (Figure 2) metabolism by this same set of rCYP2B variants indicated similar trends. This substrate is severalfold less active than DMBA, with regard to its metabolism by purified CYP2B1, and CYP2B2, with CYP2B1 being the more active isozyme (Table IV). In two sets of experiments with microsomes from infected HEPG2 cells (averaged in Table IV), the activity of microsomal rCYP2B2 is 75 times greater than the activity of microsomal rCYP2B1. The regioselectivity shown in rCYP2B2 was similar to that of the pure enzyme, both characterized by a high proportion of 4,5-dihydrodiol. The rates of turnover were also similar, although purified rCYP2B1 produced approximately 50% 4,5-dihydrodiol and 40% 3-phenol. Extremely low levels of BP-3 phenol were the only detectable products from any of the rCYP2B1 forms. Thus, all rCYP2B1 activities were over 30 times lower than for DMBA metabolism by the same preparations. Even this low activity was lost in the b_2 mutant, which was also less active in DMBA metabolism.

DMBA Metabolism by rCYP2A1 and rCYP2A2. The levels of expression of two rCYP2A isozymes were 2–4-fold lower than those for rCYP2B1 and rCYP2B2 (Table V). Both cDNA-expressed CYP2A isozymes produced DMBA metabolism with similar regioselectivity and comparable total activities. Their regioselectivity was comparable to that of CYP2B2 with metabolism oriented preferentially to the 5,6- and 12-methyl positions. Unlike rCYP2B1, but like rCYP2B2, the microsomal rCYP2A1 and rCYP2A2 activities were comparable to that of the purified reconstituted cytochrome (Table V).

Discussion

DMBA is the only substrate that provides a regioselectivity distinction between CYP2B1 and CYP2B2 (rat forms b and e) (Wilson et al., 1984), which differ by only 13 amino acids. These cytochromes produce similar proportions of 5,6- and 8,9-dihydrodiols (ratio 5/1), but CYP2B1 favors hydroxylation

at the 7-methyl position, while CYP2B2 selectively hydroxylates at the 12-methyl position (Christou et al., 1989). In this paper, we show that recombinant rat CYP2B1 and CYP2B2 expressed in HEPG2 cells exhibit the same regioselectivity differences for metabolism of DMBA as the cytochromes purified from rat liver. rCYP2B1 and rCYP2B2 retain the differences in selectivity for 7- and 12-methylhydroxylation, respectively, and also show the same preferences for formation of dihydrodiols (5,6 > 8,9; no 3,4). Although rCYP2B1 and hepatic CYP2B1 show very similar regioselectivity, there were consistent differences between rCYP2B2 and hepatic CYP2B2. Notably, rCYP2B2 exhibited a decreased ratio of 5,6- to 8,9-dihydrodiols and a redistribution from ring epoxidation to 12-methylhydroxylation. These differences may result from a minor allelic difference (or differences) in the sequence of the rCYP2B2 and the F344 hepatic CYP2B2 that have frequently been detected in rat CYP2B genes (Aoyama et al., 1989). We have seen comparable variation in regioselectivity for CYP2B1 from F344 and M520/N rats, where SDS–PAGE mobility differences suggest amino acid substitutions (Christou et al., 1989).

Previous analyses of DMBA metabolism by rat liver and lung microsomes indicate that CYP2B1 is 10–100-fold suppressed by the microsomal membrane environment relative to the metabolism by pure CYP2B1. CYP2B2, on the other hand, provides microsomal activities similar to those of the purified cytochrome (Christou et al., 1989). This difference is particularly notable since purified CYP2B1 is about 3 times more active in DMBA metabolism than purified CYP2B2, and this preference is typical for most substrates (Guengerich et al., 1982; Wolf et al., 1988; Waxman et al., 1983). The regioselectivity of DMBA metabolism by P450 in PB-induced rat liver microsomes consequently indicates a predominance of metabolism through CYP2B2, even though CYP2B1 is the major form. Data presented here (Table II) unambiguously show that rCYP2B1 expressed in HEPG2 microsomes is 20 times less active than rCYP2B2 with respect to DMBA metabolism and is about 25-fold less active than purified liver CYP2B1. In contrast, the activities of microsomal rCYP2B2 and reconstituted CYP2B2 are comparable.

Metabolism of BP by these same HEPG2 microsomes indicated an even greater suppression of rCYP2B1 activity (Table IV), while BP metabolism by purified CYP2B1 was greater than the activity of purified CYP2B2. By contrast, microsomal rCYP2B2 and purified liver CYP2B2 activities and regioselectivities were comparable for BP metabolism. This membrane suppression of CYP2B1 is not seen for all substrates and may depend on the particular membrane. Thus, rCYP2B1 expressed in HEPG2 microsomes is more active in testosterone metabolism (Aoyama et al., 1989). Benzphetamine and pentoxyresorufin are also effectively metabolized by liver microsomal CYP2B1 (Wolf et al., 1988). Recent studies in our laboratory demonstrated that the metabolism of pentoxyresorufin by phenobarbital-induced rat liver microsomes was only slightly inhibited (~20%) by the addition of DMBA (15 μM) to the microsomes. These data strongly indicate that the suppression of the catalytic activity of CYP2B1 toward DMBA in the microsomal membrane is not due to a general membrane perturbation that affects either the binding site or the interaction of CYP2B1 with oxidoreductase.

Certain amino acid substitutions in rCYP2B1 produced effects that differed between DMBA and testosterone (Figure 2) metabolism. Very different changes were produced by equivalent substitutions in rCYP2B2. These substitutions ($L^{58} \rightarrow F$, $I^{114} \rightarrow F$, and $E^{282} \rightarrow V$) are located in the NH_2-terminal half of the sequence that is identical for CYP2B1 and CYP2B2 (Figure 1; Nelson & Strobel, 1989). Introduction of an $E^{282} \rightarrow V$ substitution in rCYP2B1 (b_3 mutant) selectively enhanced DMBA metabolism while inhibiting testosterone metabolism. Although an $I^{114} \rightarrow F$ substitution in CYP2B1 (b_2 mutant) decreased both DMBA and testosterone activities, an additional $L^{58} \rightarrow F$ substitution (b_{12}) increased DMBA activity to levels much higher than even the normal rCYP2B1 activity while further lowering activity for testosterone (Figure 3B; Aoyama et al., 1989). However, these authors observed that the same double substitution resulted in a 3-fold stimulation of progesterone hydroxylation, just as we observed for DMBA (compare panels A and B of Figure 3). Thus, the additional $L^{58} \rightarrow F$ change is causing a large increase in the DMBA or progesterone-metabolizing activity while halving the rate of testosterone metabolism. Interestingly, a single $L^{58} \rightarrow F$ substitution prevents expression of rCYP2B cytochromes, suggesting a major conformational effect from the increased size and hydrophobicity of phenylalanine that can only be accommodated by introduction of phenylalanine at position 114. By contrast, in rCYP2B2 this double mutation decreased DMBA metabolism. It has recently been reported that substitution of Thr^{319} in the center of the I helix of rat CYP1A2 also produced effects that were highly substrate dependent (Furuya et al., 1989). Several substitutions that produced greatly diminished activity for benzphetamine either did not change or stimulated activity for more planar-rigid substrates (ethoxycoumarin, estradiol, and zoxazolamine).

Specific changes in steroid product formation by these same mutations (Aoyama et al., 1989) also suggest modification in the shape of the active site. Thus, the 58,114 double substitution shifts attack from the β-side of the D-ring of testosterone and androstenedione to the α-side. This change did not affect DMBA regioselectivity by CYP2B1 but substantially shifted the orientation of attack by CYP2B2. These amino acid substitutions stimulate the activity of CYP2B1 toward poor substrates that are both bulky and planar. DMBA and BP do not form a spectrally detectable complex with CYP2B1 or CYP2B2 and do not displace octylamine from the heme binding site (Marcus et al., 1985; Christou et al., 1986). Thus, the active site does not readily accommodate these polycyclic hydrocarbons, suggesting that substrate access may limit turnover. We project that access to the active site for these large rigid substrates may be more restricted for CYP2B1 when the protein is located in a microsomal membrane than when reconstituted with phosphatidylcholine. Substitutions at positions 58 and 282 in rCYP2B1 may sufficiently relieve the membrane repression of DMBA-metabolizing activity to offset inhibitory effects of the type seen for testosterone. Consistent with this view, 58,114 mutations in microsomal rCYP2B2 remain inhibitory because there is no membrane restriction of metabolism by rCYP2B2. This hypothesis is supported by the substantial stimulation of DMBA metabolism at rCYP2B1 following partial purification, while no such increase occurs for similarly treated rCYP2B2.

Expression of rCYP2A1 and rCYP2A2 proteins in HEPG2 microsomes shows that two further forms with considerable sequence homology to the CYP2B proteins resemble rCYP2B2 rather than rCYP2B1 and exhibit DMBA metabolism that is close to the activity predicted by the activity of the purified cytochrome. rCYP2A1 and rCYP2A2 have also recently been shown to exhibit the same specificity and activity for testosterone hydroxylation as the purified reconstituted cytochromes (Aoyama et al., 1990). Interestingly, the regioselectivity and

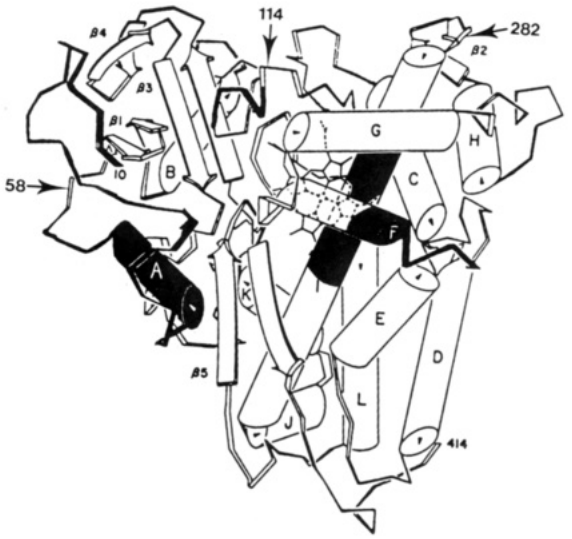

FIGURE 4: Position of CYP2B1 mutations relative to major P450 structural features [based on $P450_{CAM}$ structure of Poulos et al. (1985, 1987)]. Conserved α-helices are represented as cylinders lettered as shown in Figure 1, while arrows signify β-sheet structures. Darkened regions represent sequences reported by the membrane from antibody binding (see Figure 1) and, therefore, submerged in or adjacent to the membrane.

activities for DMBA are close to those seen for CYP2B2, suggesting similar orientation of the hydrocarbon in the active site.

Analyses of the distribution of helices and of conserved amino acids have established that the essential structural features of the soluble $P450_{CAM}$ are retained by CYP2B and CYP2A proteins. Notably, the same organization of 11 helices is retained and the amino acids that interact with the heme and O_2 are similarly located (Nelson & Strobel, 1989; Edwards et al., 1990). The three substitutions in the CYP2B sequences, together with sequence differences between CYP2B1 and CYP2B2, are indicated in Figure 1. The X-ray structure of $P450_{CAM}$ shows that most of the helices (8/11) occupy the right side, while the left side is dominated by a more open structure (Poulos et al., 1985). The active site is bordered by the heme and by hydrophobic amino acids that are located on helices B_1 and I and on the K to L linker sequence. Substitution 1 ($L^{58} \rightarrow F$) increases hydrophobicity in the center of the sequence linking helices A and B, apparently destabilizing the protein structure in a way that is compensated by the second $I^{114} \rightarrow F$ substitution. The latter is located at the beginning of the short B_1 helix in a prime position to affect the substrate binding site. The third substitution ($E^{282} \rightarrow V$) replaces a negatively charged residue with a hydrophobic residue immediately prior to helix I. Recent studies of the interaction of $P450_{CAM}$ with electron transfer proteins putaredoxin and cytochrome b_5 (Stayton et al., 1989) and of the interaction of CYP2A2 with P450 reductase (Shimuzu et al., 1991) implicate binding of these proteins to positively charged groups on the back side of the protein as it is presented in Figure 4. L^{114} also lies between two amino acid clusters that have been implicated in reductase binding and is also close to a region that may contact the substrate (Figure 1). The proximity of these key features may explain the general inhibitory effect of the single phenylalanine substitution at this position for both CYP2B1 and CYP2B2.

The nature of the interaction of CYP2B1 with the microsomal membrane is crucial for understanding the membrane suppression effect. Recent work indicates that microsomal P450 cytochromes exhibit a single major anchor to the membrane through the very hydrophobic amino-terminal sequences and that most of the protein lies outside the membrane (Edwards et al., 1990). Consistent with this view, CYP2B1 is removed from reconstituted vesicles by limited proteolysis, leaving sequence 1–21 in the liposomes (Vergeres et al., 1989). EPR analysis of the heme orientation (Kamin et al., 1985), together with the location of the amino-terminal anchor, suggest that the membrane surface lies on the near side of the structure shown in Figure 4, parallel to the plane of the paper. Major questions, however, remain concerning whether other sequences contribute to membrane binding. $P450_{scc}$ and $P450_{11\beta}$ have polar amino-terminal sequences (Edwards et al., 1990) but nevertheless adhere to membranes with high affinity. Presumably, additional hydrophobic domains that are not present in $P450_{CAM}$ contribute to the high-affinity interaction of P450 cytochromes with microsomal or mitochondrial membranes. In particular, microsomal P450 cytochromes exhibit hydrophobic domains immediately prior to helix E and at the start of helix F that are not found in $P450_{CAM}$ (Figures 1 and 4). Binding studies using antibodies raised against certain CYP2B1 peptides indicate that four sequences are selectively protected by the microsomal membrane: (A) N-terminal sequence (1–46), (B) a segment following helix B, (C) a domain surrounding the beginning of helix F, and (D) surface-facing amino acids on helix I. Recent photolabeling studies indicate interaction of lipid head groups with sequences A and D and the C-terminal region. Photoactive phospholipid acyl chains only, however, bind the membrane-spanning N-terminal sequence A (DeLemos-Chiarandini et al., 1987; Uvarov et al., 1991). This pattern of membrane protection (Figure 1) also confirms the location of the membrane parallel to the front side of the protein. Sequences B, C, and D are also proximal to, respectively, L^{58}, I^{114}, and E^{282}, which are, therefore, probably near to the membrane.

In $P450_{CAM}$, access to the substrate binding site is gained through a channel formed by Phe^{88}, Phe^{193}, and Ile^{395} (Poulos et al., 1987). A similarly located channel in CYP2B1 and CYP2B2 would open directly toward the membrane and may be altered both by phospholipid interactions and by substituents at position 114. We know from spectral data that DMBA does not readily enter the active sites of these proteins (Marcus et al., 1985). We suggest that this channel may be more readily diminished in CYP2B1 by the demands of a membrane interaction with consequent slower access by bulky substrates, like DMBA, and that amino acid substitutions at positions 114 and 282 may counteract this effect.

The relatively few differences between CYP2B1 and CYP2B2 provide an opportunity to localize amino acids involved in the membrane suppression effect. Most of the 12 amino acid differences (some differences are not found in all sequences) occur in a small region of the molecule surrounding the heme (Figure 4). These changes cause CYP2B2 to be generally less reactive, in spite of being far more readily converted to a high-spin state and chemically reduced (Wolf et al., 1988). With the exception of DMBA, these substitutions do not affect regioselectivity, suggesting that the shape of the active site is retained. On the basis of the $P450_{CAM}$ model, two substitutions between CYP2B1 and CYP2B2 may be located in the terminal sequence that flanks the substrate channel and has close proximity to the membrane (Figures 1 and 4). Further characterization of chimeric CYP2B1/CYP2B2 proteins could resolve this question.

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Registry No. DMBA, 57-97-6; benzo[*a*]pyrene, 50-32-8; cytochrome P450, 9035-51-2; 5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[*a*]anthracene, 28622-94-8; 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene, 14046-83-4; 3,4-dihydro-3,4-dihydroxy-7,12-dimethylbenz[*a*]anthracene, 72617-60-8; 7-hydroxymethyl-12-methylbenz[*a*]anthracene, 568-75-2; 7-methyl-12-hydroxymethylbenz[*a*]anthracene, 568-70-7.

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