Expression of a Human Liver Cytochrome P-450 Protein with Tolbutamide Hydroxylase Activity in *Saccharomyces cerevisiae*†

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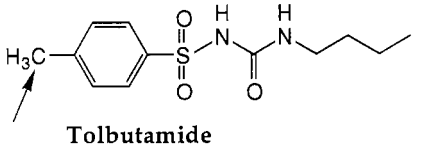
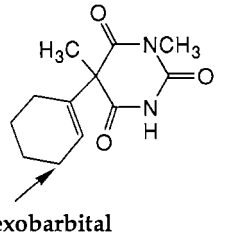
Received November 21, 1988; Revised Manuscript Received February 10, 1989

ABSTRACT: The human liver cytochrome P-450 (P-450) proteins responsible for catalyzing the oxidation of mephenytoin, tolbutamide, and hexobarbital are encoded by a multigene family (CYP2C). Although several cDNA clones and proteins related to this "P-450_{MP}" family have been isolated, assignment of specific catalytic activities remains uncertain. Sulfaphenazole was found to inhibit tolbutamide hydroxylation to a greater extent than mephenytoin or hexobarbital hydroxylation. The inhibition by sulfaphenazole was competitive for tolbutamide and hexobarbital hydroxylation but with much different K_i values (5 vs 480 μM, respectively). Inhibition of mephenytoin hydroxylase was not competitive. The results suggest that different P-450 proteins in the P-450_{MP} family may be involved in the metabolism of these compounds. A cDNA clone (MP-8) related to the P-450_{MP} family, isolated from a bacteriophage λgt11 human liver library, was expressed in *Saccharomyces cerevisiae* by using the pAAH5 expression vector. Yeast transformed with pAAH5 containing the MP-8 sequence (pAAH5/MP-8) showed a ferrous–CO spectrum typical of the P-450 proteins. Immunoblotting with anti-P450_{MP} revealed that pAAH5/MP-8 microsomes contained a protein with an M_r similar to that of P-450_{MP-1} (~48 000) that was not present in microsomes from yeast transformed with pAAH5 alone (1.7×10^4 molecules of the expressed P-450 per cell). Microsomes from pAAH5/MP-8 contained no detectable mephenytoin 4′-hydroxylase activity but were more active in tolbutamide hydroxylation, on a nanomoles of P-450 basis, than human liver microsomes. The pAAH5/MP-8 microsomes also contained hexobarbital 3′-hydroxylase activity, although the enrichment compared to liver microsomes was not great with respect to the tolbutamide hydroxylase activity. Thus, the hydroxylation of mephenytoin and tolbutamide is catalyzed by similar but distinct P-450s. P-450_{MP-1} and P-450_{MP-2} contain mephenytoin hydroxylase activity. The MP-8 cDNA clone encodes a protein with tolbutamide hydroxylase activity, now termed P-450_{TB}.

The cytochrome P-450[1] catalyzed oxidation of the drug mephenytoin shows genetic polymorphism in humans (Küpfer & Preisig, 1984; Wedlund et al., 1984). Two proteins, P-450_{MP-1} and P-450_{MP-2}, have been isolated from human liver on the basis of their (*S*)-mephenytoin 4′-hydroxylase activity (Shimada et al., 1986). Evidence has also been presented that P-450_{MP-1} and P-450_{MP-2} or closely related P-450s may catalyze tolbutamide methyl hydroxylation and hexobarbital 3′-hydroxylation (Knodell et al., 1987, 1988). These activities have also been reported to show polymorphic behavior in humans (Scott & Poffenbarger, 1979; Knodell et al., 1988). Several similar but distinct P-450 proteins related to P-450_{MP-1} have been isolated (Shimada et al., 1986; Lasker et al., 1987; Wrighton et al., 1987; Ged et al., 1988) and are classified as being members of the P-450_{MP} family (formerly P-450IIC, now CYP2C genes; Nebert et al., 1987, 1989). Three distinct cDNA clones classified in the P-450_{MP} family have been isolated by several laboratories (Umbenhauer et al., 1987; Kimura et al., 1987a; Okino et al., 1987; Yasumori et al., 1987; Ged et al., 1988). On the basis of sequence data of proteins and cDNA clones isolated to date, members of the P-450_{MP} family can be categorized into two groups having 85% sequence similarity (Ged, 1988). However, the assignment of specific catalytic activities to individual proteins within these groups is still not possible.



Tolbutamide



Hexobarbital

Uncertainty still exists whether (*S*)-mephenytoin 4′-hydroxylation and tolbutamide methyl hydroxylation are catalyzed by the same or by distinct but highly related P-450s. Knodell et al. (1987) reported that anti-P-450_{MP-1} inhibited

† This research was supported in part by USPHS Grants CA 44353, ES 00267, and ES 07028 from the National Institutes of Health. F.P.G. was a Burroughs Wellcome Scholar in Toxicology (1983–1988) while this work was carried out.

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[1] Abbreviations: P-450, cytochrome P-450; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid. For further discussion of the proteins termed P-450_{MP-1}, P-450_{MP-2}, and P-450_{MP-3} see Ged et al. (1988). Uncertainty still exists as to the assignment of specific genes to these proteins; in the original classification of Nebert et al. (1987) the genes are grouped in the P-450IIC 8 and 9 families (P-450_{MP-1} and P-450_{MP-2} would actually be P-450IIC 9 and P-450_{MP-3} would be P-450IIC 8). In the new classification, P-450_{MP-3} should be CYP2C 8, P-450_{TB} should be CYP2C 10, and P-450_{MP-1} and P-450_{MP-2} may be in the CYP2C 9 group. For discussion of other human P-450 enzymes and their properties, see Guengerich (1989).

0006-2960/89/0428-4993$01.50/0

microsomal tolbutamide oxidation. Mephenytoin acted as a competitive inhibitor of tolbutamide hydroxylation, and tolbutamide was a competitive inhibitor of mephenytoin oxidation. However, Miners et al. (1988) reported that mephenytoin only weakly inhibited tolbutamide hydroxylation, suggesting that distinct P-450 proteins might be involved in the oxidation of these two compounds. Humans phenotyped as poor or extensive metabolizers of mephenytoin showed no difference in tolbutamide oxidation (Knodell et al., 1987).

A better understanding of the individual genes and proteins in the P-450$_{MP}$ family is necessary to understand the biochemical basis of the observed variations in drug oxidation. In this paper, sulfaphenazole, a potent inhibitor of tolbutamide hydroxylase (Miners et al., 1988), was used as a probe in the question of whether the same P-450 is involved in the oxidation of both mephenytoin and tolbutamide. Also, a yeast-based expression system was developed by using a cDNA clone (MP-8) related to the P-450$_{MP}$ family (Umbenhauer et al., 1987). The P-450 encoded by cDNA clone MP-8 in the yeast showed tolbutamide methyl hydroxylase and hexobarbital 3′-hydroxylase activities but not (*S*)-mephenytoin 4′-hydroxylase activity.

Experimental Procedures

Microorganisms. *Escherichia coli* strain MC1060 was grown on LB media [1% bactopeptone (w/v), 0.5% yeast extract (w/v, Difco, Detroit, MI), and 1% NaCl (w/v)]. *Saccharomyces cerevisiae* strain D12 (cir$^+$, leu$^-$; gift from Genex Corp., Gaithersburg, MD) was cultivated on YPD media [2% bactopeptone (w/v), 1% yeast extract (w/v, Difco), and 2% glucose (w/v)]. Transformed yeast were maintained on minimal YNBD media [0.67% yeast nitrogen base without amino acids (w/v, Difco) and 2% glucose (w/v)].

pAAH5/MP-8 Vector Construction. As reported previously (Umbenhauer et al., 1987), rabbit polyclonal antisera raised against purified P-450$_{MP-1}$ were used to select a clone termed MP-8 from a human liver library constructed in bacteriophage λgt11. A 1.6-kb *Eco*RI fragment of MP-8 was subcloned in M13mp9.

The yeast expression vector containing the MP-8 clone was constructed as shown in Figure 1. Digestion of M13mp9 containing MP-8 with a combination of *Sac*I and *Eco*RI endonucleases (New England Biolabs, Beverly, MA) released a 1.5-kb fragment that contained all but 69 nucleotides on the 5′ end of the coding region of MP-8. Oligonucleotides were synthesized by using the β-phosphoramidite method on a Biosearch cyclone synthesizer (Biosearch, San Rafael, CA). These were used to construct linkers to ligate MP-8 into the yeast expression vector pAAH5 (gift from Dr. B. Hall, University of Washington, Seattle) at the *Hin*dIII site in the plasmid. The 5′ linker contained *Hin*dIII and *Sac*I sites and was designed to replace the nucleotides in the coding region between the ATG initiation codon and the *Sac*I site. The N-terminal amino acid sequence of purified P-450$_{MP-1}$ (Shimada et al., 1986) was used as a guide to supply the 15 nucleotides missing from the 5′ end of the MP-8 clone as it was originally isolated (Umbenhauer et al., 1987). An *Eco*RI/*Hin*dIII linker allowed ligation of the 3′ end of the MP-8 clone to pAAH5. Complementary oligomers for the 5′ linker were 77 and 69 nucleotides in length. The 3′ linker was made from oligomers of 15 and 22 nucleotides. The 5′ 77-mer and the 3′ 22-mer were phosphorylated in the same tube by using polynucleotide kinase (New England Biolabs) and then extracted with phenol to inactive the kinase. The 69-mer and 15-mer oligomers were added, followed by extraction with phenol/$CHCl_3$ and then precipitation with C_2H_5OH. Before use, the oligomers were resuspended in water, heated to 95 °C for 3 min, and allowed to cool slowly to 25 °C to anneal into double-stranded linkers. The pAAH5 vector was linearized with *Hin*dIII (New England Biolabs) and treated with bacterial alkaline phosphatase (Bethesda Research Labs, Gaithersburg, MD) to prevent religation. The MP-8 insert, pAAH5 vector, and linkers were ligated in one step by using T4 DNA ligase (New England Biolabs). *E. coli* MC1060 cells (made competent with $CaCl_2$) were transformed with the ligation mixture and plated on LB media containing ampicillin (100 μg mL^{-1}; Sigma Chemical Co., St. Louis, MO). Colonies were screened for pAAH5 containing the MP-8 clone with a ^{32}P-end-labeled oligomer (20-mer) previously used for sequencing the clone. Several positive colonies were selected and vectors isolated by using a quick boiling method (Holmes & Quigley, 1981). *Bam*HI restriction analysis was used to determine the vectors with MP-8 in the correct orientation behind the ADH1 promoter. The correct constructs were used to transform yeast according to the method of Beggs (1978), except that lyticase (Sigma) rather than a snail gut extract was used to prepare spheroplasts. Yeast cells transformed with pAAH5 or pAAH5 containing the MP-8 clone were selected by growth in regeneration agar [1.2 M sorbitol, 2% glucose (w/v), 0.67% yeast nitrogen base without amino acids (w/v), and 3% agar (w/v, Difco)]. The two yeasts are referred to as pAAH5 and PAAH5/MP-8 (or K3A) in the text.

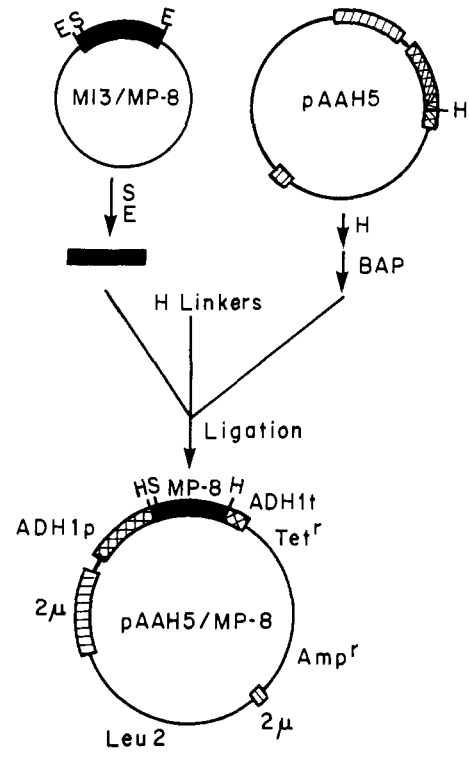

FIGURE 1: Construction of pAAH5/MP-8 expression plasmid. Complete details are presented under Experimental Procedures. The solid box indicates the MP-8 coding region. The hatched boxes represent the promoter and terminator of the pAAH5 vector. The lined boxes indicate yeast 2 μ DNA. Indicated restriction sites and restriction endonucleases are (E) *Eco*RI; (S) *Sac*I; (H) *Hin*dIII. "BAP" indicates bacterial alkaline phosphatase.

Yeast Microsomes. Microsomes were prepared as previously described (Oeda et al., 1985) with modifications for lysing the cells. Yeast harvested during logarithmic growth ($A_{600} \sim 1.6$) were incubated with yeast lytic enzyme (ICN Biomedicals, Cleveland, OH) at a concentration of 5 mg mL^{-1} in 10 mM Tris-HCl buffer (pH 7.5) containing 2 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.4 mM phenylmethanesulfonyl fluoride at 30 °C for 60 min. Cells were washed and

resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.65 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.4 mM phenylmethanesulfonyl fluoride and then lysed by sonication (6 15-s bursts at 140 W with the 0.5-in. probe of a Sonifier cell disruptor, Model W185; Heat Systems-Ultrasonics, Plainview, NY). Microsomes were prepared by first centrifuging the suspension at 10000*g* for 20 min (discarding the pellet) and then centrifuging at 125000*g* for 90 min to obtain a pellet. This microsomal pellet was homogenized in 0.1 M potassium pyrophosphate buffer (pH 7.4) containing 1 mM EDTA and 20 μM butylated hydroxytoluene and centrifuged at 125000*g* for 60 min. The pellet was resuspended in 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol.

HPLC Assays. The enzymatic hydroxylations of tolbutamide, hexobarbital, and mephenytoin were monitored by using the general incubation conditions and HPLC assays previously described (Shimada et al., 1986; Knodell et al., 1987, 1988). In most of the experiments presented here, the 4′-hydroxylation of (*S*)-[*methyl*-^{14}C]mephenytoin was monitored by passing the effluent of the HPLC column into a Flo-one scintillation counter (Radiomatic, Tampa, FL), where it was continuously mixed with Radiomatic Flo-scint II (Radiomatic) cocktail in a ratio of 1:3 (v/v). In some of the experiments involving the hydroxylation of tolbutamide and hexobarbital, the effluent passed into the flow cell of a Hewlett-Packard 8540 diode array spectrophotometer and both chromatographic traces and absorbance spectra were recorded. Incubations with [1,3,6-^{3}H]benzo[*a*]pyrene (500 mCi mmol^{-1}) were done essentially as described elsewhere (Gozukara et al., 1982) by using a similar HPLC system. The effluent of the column was also passed through the Flo-one scintillation counter. Authentic standards of 3- and 9-hydroxybenzo[*a*]-pyrene and benzo[*a*]pyrene 4,5-, 7,8-, and 9,10-dihydrodiols and 1,6-, 3,6-, and 6,12-diones were obtained from Dr. L. J. Marnett (Wayne State University, Detroit, MI) or from the National Cancer Institute Chemical Repository through Midwest Research Institute (Kansas City, MO).

Other Assays. Immunoblotting analysis with anti-P-450$_{MP}$ was used to assay P-450 expression in yeast transformed with pAAH5/MP-8. Several transformed yeast colonies were grown overnight in YPD media to saturation. Yeast cells were pelleted by centrifugation at 8000*g* for 5 min, washed once, and resuspended in water. Cell extracts were prepared by boiling cells in 6 mM Tris-HCl buffer (pH 6.8) containing 1% NaDodSO$_4$ (w/v), 5% 2-mercaptoethanol (w/v), 8% glycerol (v/v), and 0.07% pyronin Y (w/v), followed by centrifugation (5000*g* for 5 min) to pellet debris. Supernatants were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis (Laemmli, 1970), transferred to sheets of nitrocellulose, and developed with rabbit anti-P-450$_{MP}$ as previously described (Guengerich et al., 1982; Shimada et al., 1986). Yeast and human liver microsomes were analyzed by using the same procedure.

Reduced CO difference spectra of P-450 in whole yeast cells (approximately 1.5 × 10^9 cells mL^{-1}) were obtained as described for yeast by the procedure of Oeda et al. (1985), based on the method of Omura and Sato (1964). Yeast cells were grown to saturation, washed in water, and resuspended in 0.1 M potassium phosphate buffer (pH 7.4) prior to analysis. Spectra were obtained with a Cary 219 spectrophotometer (Varian, Walnut Creek, CA) in the automatic base-line correction mode.

Results

Sulfaphenazole Inhibition. Sulfaphenazole has been reported to be an effective inhibitor of tolbutamide hydroxylation both in human liver microsomes and in vivo (Pond et al., 1977; Miners et al., 1988). The inhibitory effects of sulfaphenazole on mephenytoin, tolbutamide, and hexobarbital hydroxylation in human liver microsomes were determined. Sulfaphenazole was a potent inhibitor of tolbutamide hydroxylase; however, mephenytoin and hexobarbital hydroxylation were not inhibited to nearly so great an extent (Figure 2). The nature of sulfaphenazole inhibition also differed. Sulfaphenazole appears to be a competitive inhibitor of tolbutamide hydroxylase (Figure 3A). The inhibition of hexobarbital hydroxylation also showed some competitive character (Figure 3B) though the apparent K_i (~480 μM) was significantly higher than in the case of tolbutamide hydroxylation (~5 μM). The inhibition of mephenytoin 4′-hydroxylation was rather nondescript and not competitive at all in the example shown in Figure 3C and other steady-state kinetic experiments done with this and other liver samples.

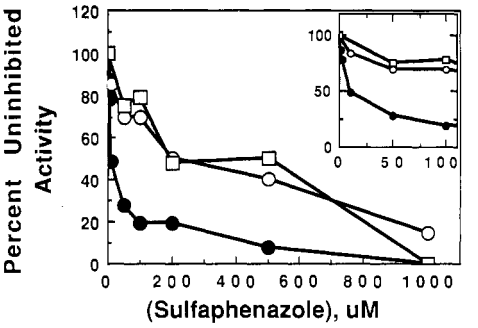

FIGURE 2: Inhibition of hydroxylase activities by sulfaphenazole in human liver microsomes. Experiments were done with human liver sample HL 117; uninhibited rates of hydroxylation of tolbutamide (●), hexobarbital (□), and mephenytoin (○) were 0.60, 0.39, and 0.05 nmol of product formed min^{-1} (nmol of P-450)$^{-1}$, respectively.

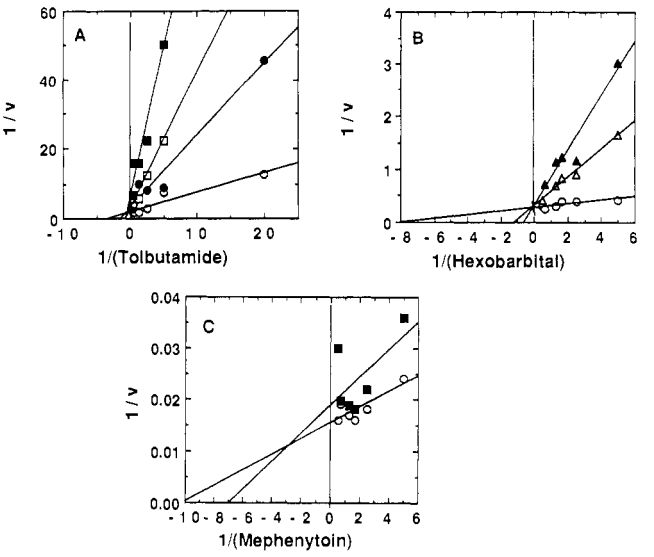

FIGURE 3: Analysis of inhibition of hydroxylase activities by sulfaphenazole in human liver microsomes. The experiments were done with the human liver sample HL 117 as in Figure 2. Reaction velocity (v) is expressed in nmol of product formed min^{-1} (nmol of P-450)$^{-1}$. (A) Inhibition of tolbutamide hydroxylation. Sulfaphenazole concentrations were 0 (○), 0.005 (●), 0.010 (□), and 0.050 mM (■). The estimated K_i for sulfaphenazole was 5 μM. (B) Inhibition of hexobarbital 3′-hydroxylation by sulfaphenazole. Sulfaphenazole concentrations were 0 (○), 0.2 mM (△), and 0.5 mM (▲). The estimated K_i for sulfaphenazole was 0.48 mM. (C) Inhibition of mephenytoin 4′-hydroxylation by sulfaphenazole. Sulfaphenazole concentrations were 0 (○) and 1.0 mM (■). The lines were drawn by using linear regression analysis of the points in the double-reciprocal plots.

Table I: Catalytic Activities of Yeast and Human Liver Microsomes

microsomal preparation	nmol of product formed min^{-1} (nmol of P-450)$^{-1}$ [a]				
	tolbutamide methyl hydroxylation	hexobarbital 3′-hydroxylation	(*S*)-mephenytoin 4′-hydroxylation	benzo[*a*]pyrene hydroxylation	
				all products	3-hydroxylation
human liver HL 102	0.52	0.20	0.10	0.024	0.0037
human liver ($\bar{X}$, n = 14)	0.65[b]	0.38[c]	0.16[d]	0.030[e]	0.0037[f]
yeast pAAH5/MP-8	5.50	0.34	<0.01	<0.002	<0.001
pAAH5/MP-8, -NADPH	<0.01	<0.01	<0.01	<0.002	<0.001
pAAH5	<0.01	<0.01	<0.01	<0.002	<0.001

[a] Incubations were generally done with 0.25–0.5 nmol of P-450 for 15–25 min at 37 °C. [b] Range 0.1–1.8 [from Knodell et al. (1987)]. [c] Range 0.04–0.94 [from Knodell et al. (1988)]. [d] Range 0.01–0.46. [e] Range 0.004–0.12. [f] Range 0.0017–0.014.

Yeast Expression. By use of the strategy outlined in Figure 1, the complete coding region of cDNA clone MP-8 was placed in the expression vector pAAH5, under control of the ADH1 promoter. The uniquely designed 5′-linker not only allowed ligation of the MP-8 clone to pAAH5 at the *Hin*dIII site but also supplied the five codons missing from the clone as it was originally isolated from the human liver cDNA library (Umbenhauer et al., 1987).

Colonies of yeast transformed with pAAH5 alone and pAAH5 containing MP-8 were screened for expression of P-450 by immunoblot analysis, using rabbit polyclonal antisera to P-450$_{MP-1}$ (data not shown). One colony, termed pAAH5/MP-8 or K3A, was selected for further studies. Microsomes prepared from yeast harboring pAAH5/MP-8 contained a protein recognized by anti-P450$_{MP}$ with the same apparent M_r as P-450$_{MP-1}$ (Figure 4). This protein was not present in pAAH5. The identities of the other bands recognized by anti-P-450$_{MP-1}$ in pAAH5 and pAAH5/MP-8 microsomes are unknown (they did not appear in liver). The Fe^{2+}–CO vs Fe^{2+} difference spectra of pAAH5/MP-8 cells showed a peak at 450 nm, indicative of P-450 proteins, that was not observed in cells transformed with pAAH5 alone (Figure 5). pAAH5/MP-8 contains approximately 1.7×10^4 molecules of P-450/cell, with more than 90% of this being the expressed protein corresponding to the MP-8 sequence. A similar estimate was obtained by comparing immunoblots of pAAH5/MP-8 cell extracts and known amounts of purified P-450$_{MP-1}$ by densitometry. Both pAAH5/MP-8 and pAAH5 exhibited a peak at 420 nm that has been reported by others (Yoshida et al., 1974; Oeda et al., 1985). Most, if not all, of this peak is apparently due to uncharacterized CO-binding hemoproteins endogenous to the yeast and not to cytochrome P-420.

Catalytic Activities. Since the MP-8 cDNA was originally selected from the bacteriophage λgt11 library by using antisera raised to P-450$_{MP-1}$, it was thought that this clone might encode a protein with mephenytoin 4′-hydroxylase activity. However, microsomes prepared from pAAH5/MP-8 did not exhibit this activity when assayed with (*S*)-[*methyl*-^{14}C]mephenytoin (Figure 6, Table I). The absence of mephenytoin hydroxylase in pAAH5/MP-8 was confirmed by using a thin-layer radiochromatographic procedure (Shimada et al., 1985; data not shown).

Previous experiments have suggested that a member of the P-450$_{MP}$ family contains tolbutamide methyl hydroxylase activity (Knodell et al., 1987). Microsomes from pAAH5/MP-8 showed high tolbutamide hydroxylase activity, whereas pAAH5 microsomes showed none (Figure 7). The basic conclusions that the pAAH5/MP-8 yeast have high tolbutamide hydroxylase activity and no detectable mephenytoin 4′-hydroxylase activity were confirmed with two additional preparations of yeast. The UV spectrum of the putative hydroxymethyl tolbutamide formed by pAAH5/MP-8 was identical with those of an authentic standard and the product formed in human liver microsomes (Figure 8). The addition of sulfaphenazole (5 μM) reduced hydroxytolbutamide for-

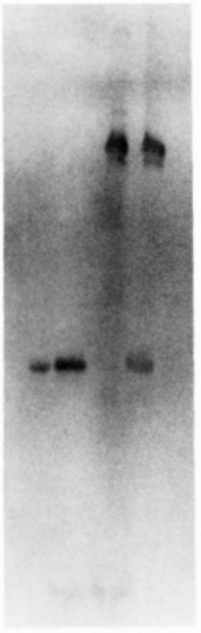

FIGURE 4: $NaDodSO_4$ electrophoresis and immunochemical blotting of human liver and yeast microsomal proteins. Samples were electrophoresed on 7.5% (w/v) polyacrylamide gels and blotted with rabbit polyclonal anti-P-450$_{MP}$. (Lane 1) HL 102 microsomes, 5 pmol of P-450; (lane 2) purified P-450$_{MP-1}$ and P-450$_{MP-2}$, 2 pmol of P-450; (lane 3) pAAH5 microsomes, ~0.3 pmol of P-450; (lane 4) pAAH5/MP-8 microsomes, 4 pmol of P-450. An equal amount of microsomal protein was used for the pAAH5 and pAAH5/MP-8 samples.

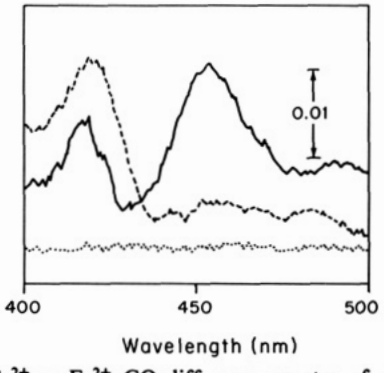

FIGURE 5: Fe^{2+} vs Fe^{2+}–CO difference spectra of pAAH5- and pAAH5/MP-8-containing yeast. Yeast cells were used to obtain spectra as described under Experimental Procedures: corrected base line was obtained with cells in buffer only (···); Fe^{2+}–CO spectrum of pAAH5-containing cells (- -); Fe^{2+}–CO spectrum of pAAH5/MP-8-containing cells (—).

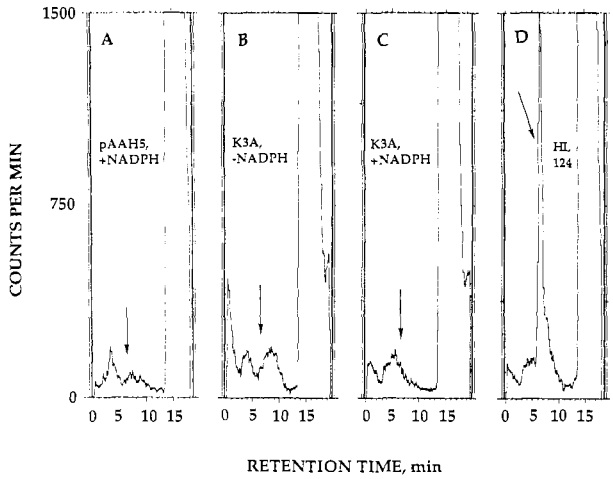

FIGURE 6: Assays of (*S*)-mephenytoin 4′-hydroxylase activity in human liver and yeast microsomes. Microsomes from pAAH5, K3A (pAAH5/MP-8) (75 pmol of P-450), or HL 124 were incubated with (*S*)-[*methyl*-^{14}C]mephenytoin (1 mM; 16.2 mCi mmol^{-1}) for 30 min at 37 °C with or without NADPH. With pAAH5 microsomes, an amount of protein equivalent to that in the K3A sample was used. Products were extracted with CH_2Cl_2, dried under N_2, and dissolved in CH_3OH. Aliquots were injected onto a 5 μm 4.6 mm i.d. × 25 cm Ultrasphere-ODS C_{18} HPLC column (Altex, Berkeley, CA) and eluted with 36% CH_3OH in H_2O (v/v) (flow rate 1.0 mL min^{-1}); the effluent was passed through a Radiomatic Flo-one scintillation counter. (A) pAAH5 microsomes; (B) K3A microsomes without NADPH; (C) K3A microsomes with NADPH; (D) HL 124 microsomes. The arrow shows the retention time of standard 4′-hydroxymephenytoin.

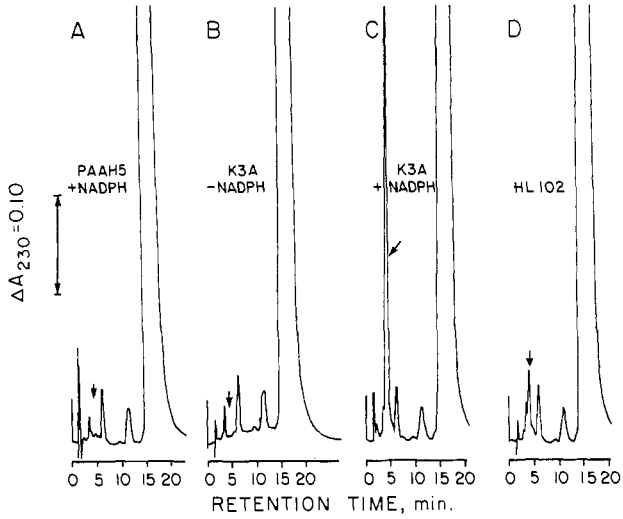

FIGURE 7: Tolbutamide methyl hydroxylase assays of human liver and yeast microsomes. Tolbutamide (2.5 mM) was added to yeast microsomes from pAAH5 (~20 pmol of P-450) and K3A (pAAH5/MP-8) (250 pmol of P-450) or to HL 102 microsomes (500 pmol of P-450) with or without NADPH. Equal amounts of microsomal protein were taken for pAAH5 and K3A. Following incubation at 37 °C for 15 min, samples were extracted, dried, and redissolved in CH_3OH as in Figure 6. Assays were done by using the HPLC column described in Figure 6 but with a different isocratic buffer system (12% CH_3CN, 0.04% H_3PO_4, 88% H_2O, v/v/v). (A) pAAH5 microsomes; (B) K3A microsomes without NADPH; (C) K3A microsomes with NADPH; (D) HL 102 microsomes. The retention time of standard hydroxymethyl tolbutamide is shown by the arrow.

mation to 45% of the uninhibited rate (cf. Figure 2). Omission of NADPH from the reaction mixtures eliminated product formation (Table I).

A previous paper from this laboratory suggested that the same P-450 may be responsible for the hydroxylation of both mephenytoin and hexobarbital (Knodell et al., 1988). Therefore, it was of interest to determine if the protein encoded

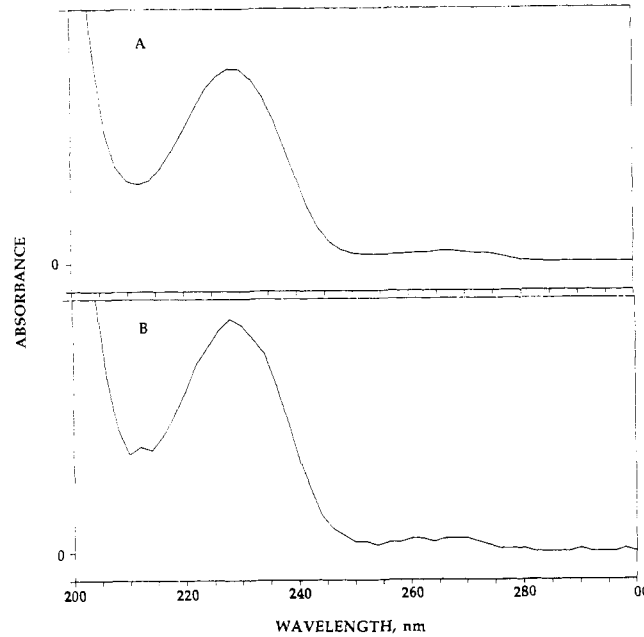

FIGURE 8: UV spectra of standard 3′-hydroxymethyl tolbutamide and product formed in K3A yeast microsomes. Microsomes were assayed for tolbutamide hydroxylase activity as described in Figure 7. Authentic hydroxymethyl tolbutamide or microsomal extract was passed through the HPLC column into the diode array spectrophotometer. UV spectra were taken on the material eluting from the column at 4.5 min, the retention time of standard hydroxymethyl tolbutamide. (A) Authentic 3′-hydroxymethyl tolbutamide (full scale 0.15); (B) hydroxymethyl tolbutamide formed by K3A (pAAH5/MP-8) microsomes (full scale 0.01).

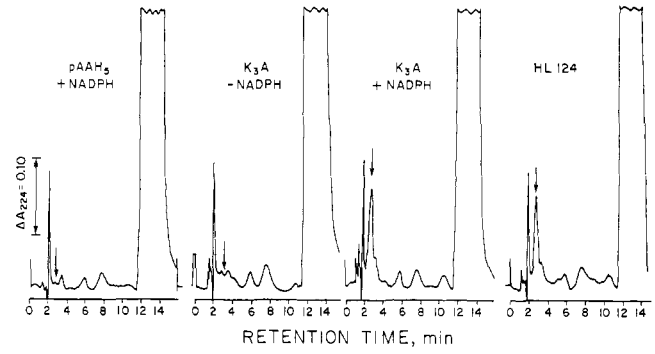

FIGURE 9: Hexobarbital 3′-hydroxylase assays of human liver and yeast microsomes. Microsomes from pAAH5 and pAAH5/MP-8 yeasts and liver HL 124 (same amounts as used for HL 102 microsomes in Figure 7) were incubated with hexobarbital (1 mM) with or without NADPH for 15 min at 37 °C. Products were extracted, dried, and redissolved in CH_3OH as in Figure 6. Assays were done as described in Figure 7. (A) pAAH5 microsomes; (B) K3A microsomes minus NADPH; (C) K3A microsomes with NADPH; (D) HL 124 microsomes. The arrow designates the retention time of standard 3′-hydroxyhexobarbital.

by the MP-8 clone has hexobarbital 3′-hydroxylase activity. Microsomes from pAAH5/MP-8, but not from pAAH5, catalyzed the hydroxylation of hexobarbital (Figure 9). The identity of 3′-hydroxyhexobarbital formed by pAAH5/MP-8 was confirmed by comparison of the UV spectra of the yeast product with authentic 3′-hydroxyhexobarbital standard and the product formed in human liver microsomes (data not shown).

Very recently Yasumori et al. (1988) reported the expression of a cDNA clone very similar to MP-8 (P-450 human 2; two nonsilent nucleotide differences) using a different vector. The expressed proteins were not examined for ability to hydroxylate tolbutamide, hexobarbital, or mephenytoin, but the authors found low benzo[*a*]pyrene hydroxylase activity as judged by

fluorescence measurements. pAAH5/MP-8 microsomes did not oxidize benzo[*a*]pyrene (Table I).

Discussion

The isolation and sequencing of a cDNA clone (MP-8) related to P-450 mephenytoin 4′-hydroxylase was previously reported from this laboratory (Umbenhauer et al., 1987). This MP-8 sequence was expressed in yeast by using the pAAH5 expression vector. Immunoblotting revealed that the pAAH5/MP-8 construct encodes a full-length P-450 protein, $M_r \sim 48000$, similar in size to P-450$_{MP-1}$ and P-450$_{MP-3}$ (Ged et al., 1988). The yeast is capable of inserting the heme moiety, as evidenced by the characteristic P-450 Fe^{2+}–CO spectrum and the catalytic activity measurements. The level of expression of MP-8 ($\sim 1.7 \times 10^4$ molecules/cell) is somewhat low compared to levels reported by others for different P-450 clones [i.e., Oeda et al. (1985) reported 4–8 $\times 10^5$ molecules of rat liver P-450MC/yeast cell when transformed with pAMC1 cDNA clone]. The difference in efficiency may be due to use of different yeast strains (Sakaki et al., 1985). Also, some clones are known to be expressed at higher levels than others; alternatively, the expressed mRNAs or proteins may be more stable in some instances. Rat P-450 clones that were mutagenized to produce a P-450 protein differing in a single amino acid from the original were found to not be expressed as well as the parent protein in yeast or to produce a less stable protein (Imai & Nakamura, 1988; Shimuzu et al., 1988). The reasons for these differences between clones are unknown, but the MP-8 clone may be one of those exhibiting a lower level of expression.

The MP-8 cDNA was expected to possibly encode a protein with mephenytoin 4′-hydroxylase activity, as the MP-8 clone was originally selected from a cDNA library with antisera raised to P-450$_{MP-1}$ and the deduced amino acid sequence of the clone matched the N-terminal amino acid sequences of purified P-450$_{MP-1}$ and P-450$_{MP-2}$ (Shimada et al., 1986; Umbenhauer et al., 1987). However, no mephenytoin 4′-hydroxylase activity could be detected in pAAH5/MP-8 microsomes; the limit of detection was far below the levels measured in most human liver microsomal samples. Rather, sequence MP-8 encodes a P-450 with tolbutamide methyl hydroxylase activity. The pAAH5/MP-8 microsomes were more active in tolbutamide hydroxylation, on a nanomoles of P-450 basis, than any of the human liver microsomal samples examined (Table I).

The selective and competitive inhibition of tolbutamide hydroxylation by sulfaphenazole (Figures 2 and 3) supports the view that the mephenytoin 4′-hydroxylation and tolbutamide methyl hydroxylation are catalyzed by distinct P-450s. Since P-450$_{MP-1}$ and P-450$_{MP-2}$ appear to be true mephenytoin 4′-hydroxylases and P-450$_{MP-3}$ (which appears to correspond to a sequence 15% different than that coded by cDNA MP-8; Ged et al., 1988) has activity toward neither tolbutamide nor mephenytoin, we term the protein expressed from the MP-8 clone P-450$_{TB}$. The identification of a P-450$_{TB}$ protein distinguished from P-450$_{MP}$ using classical purification techniques, immunoreactivity, or catalytic activity has not yet been realized. Results suggesting that P-450$_{MP-1}$ and P-450$_{MP-2}$ have tolbutamide hydroxylase activity (i.e., copurification of activities, immunoinhibition, and competitive inhibition of tolbutamide hydroxylase by mephenytoin) are attributed to the structural similarities of the two proteins. It remains to be determined if P-450$_{MP-1}$ or P-450$_{MP-2}$ contains any tolbutamide hydroxylase activity, as the observed catalytic activity toward tolbutamide (Knodell et al., 1987) could possibly be due to contamination by P-450$_{TB}$.

Blotting of genomic DNA from human lymphocytes using P-450$_{MP}$ probes indicated there are at least seven gene-related sequences (genes or pseudogenes) in the P-450$_{MP}$ family (Ged et al., 1988). However, in this laboratory only three distinct cDNA clones (MP-4, MP-8, and MP-20) have been isolated from a human library (constructed from a single liver) by using antisera or oligonucleotide probes related to P-450$_{MP}$ (Umbenhauer et al., 1987; Ged et al., 1988) (MP-12 is very closely related to MP-20 and might not be a distinct gene product). Other laboratories have reported the isolation of one or more of these three clones (Kimura et al., 1987a; Okino et al., 1987; Yasumori et al., 1987), but additional clones have not been identified. The MP-20 clone appears to encode P-450$_{MP-3}$, which apparently has neither mephenytoin, hexobarbital, nor tolbutamide hydroxylase activity (Ged et al., 1988). Thus, at present, the MP-4 clone (P-450 human-2; IIC9) is the only remaining known candidate for mephenytoin hydroxylase activity in human liver. The primary structures of the proteins encoded by sequences MP-4 and MP-8 differ only in two amino acids, Cys-358 vs Tyr-358 and Asp-417 vs Gly-417, respectively. These are relatively major changes as far as side-chain reactivities are concerned. Others have reported that a change of one or two amino acids in a P-450 protein can drastically reduce catalytic activity (Kimura et al., 1987b; Imai & Nakamura, 1988; Ishida et al., 1988; Shimuzu et al., 1988). However, the effect of changing one or two amino acids on the catalytic specificity of a P-450 has not been studied in great detail yet. The yeast expression system is currently being used to determine if MP-4 encodes mephenytoin 4′-hydroxylase activity.

Recent evidence from this laboratory strongly suggests that P-450$_{MP}$ catalyzes hexobarbital 3′-hydroxylation (Knodell et al., 1988)—i.e., copurification of activities, inhibition of hexobarbital hydroxylation by anti-P-450$_{MP}$, correlation of the two activities in different liver samples, and competitive inhibition of hexobarbital hydroxylase by mephenytoin and mephenytoin hydroxylase by hexobarbital in vitro. Also, in vivo hexobarbital clearance appears to segregate with the mephenytoin hydroxylation polymorphism in limited studies. The present study indicates that P-450$_{TB}$ has hexobarbital hydroxylase activity and not mephenytoin hydroxylase activity. The initial results regarding sulfaphenazole inhibition (Figure 2) would suggest that tolbutamide and hexobarbital are hydroxylated by different P-450s. However, the plots in Figure 3B suggest some competitive nature of the inhibition. Further, as the ratios of yeast/human catalytic activities are considered in Table I, it is clear that the level of hexobarbital hydroxylase activity in the yeast is not so high as would be expected on the basis of tolbutamide hydroxylase activity. It would appear that P-450$_{MP-1}$, P-450$_{MP-2}$, and P-450$_{TB}$ are capable of metabolizing hexobarbital, possibly reflecting a high degree of structural similarity between these proteins. The confirmation of this hypothesis awaits either the complete isolation of P-450$_{MP-1}$ or P-450$_{MP-2}$ from other P-450s or the expression of P-450$_{MP-1}$ and P-450$_{MP-2}$ genes in a vector system.

Finally, the regulation of the related proteins may be considered. In vivo studies indicate that hydroxylase activity toward tolbutamide, hexobarbital, and mephenytoin is induced by treatment with rifampicin or barbituates (Zilly et al., 1975; Zhou et al., 1988). In fetal tissue none of these catalytic activities (or P-450$_{MP}$-related proteins or mRNAs) are expressed. Familial inheritance has been observed for mephenytoin hydroxylation, and this phenomenon may be linked to hexobarbital hydroxylation (Knodell et al., 1988) but does not appear to be linked to tolbutamide hydroxylation (Knodell

et al., 1987). The utility of an expression system is obvious for studying individual clones and proteins in the P-450$_{MP}$ family. Through studies of this type the biochemical mechanisms underlying the genetic polymorphism related to the P-450$_{MP}$ family may be better understood.

ACKNOWLEDGMENTS

We thank S. T. Wright for oligonucleotide synthesis and C. G. Turvy for technical assistance with preparation of yeast enzyme.

Registry No. Tolbutamide, 64-77-7; hexobarbital, 56-29-1; (*S*)-mephenytoin, 70989-04-7; benzo[*a*]pyrene, 50-32-8; sulfaphenazole, 526-08-9; (*S*)-mephenytoin 4′-hydroxylase, 96779-46-3; tolbutamide methyl hydroxylase, 106527-94-0; cytochrome P-450, 9035-51-2; hexobarbital 3′-hydroxylase, 120544-83-4.

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