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Separation of Human Liver Microsomal Tolbutamide Hydroxylase and (S)-Mephenytoin 4′-Hydroxylase Cytochrome P-450 Enzymes

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SUMMARY

Purification and immunoinhibition studies have suggested that the hydroxylations of (*S*)-mephenytoin and tolbutamide are catalyzed by rather similar forms of human liver cytochrome P-450 (P-450). However, the two activities are not well correlated *in vivo*; sulfaphenzaole is a selective inhibitor of tolbutamide hydroxylation, and expression of P-450 2C10 cDNA in yeast yields a protein that hydroxylates tolbutamide but not (*S*)-mephenytoin. The P-450 2C8, 2C9, and 2C10 cDNAs have all been isolated, and their sequences are known to be closely related (>80%). Highly sensitive radiochromatographic assays were set up, and tolbutamide and (*S*)-mephenytoin hydroxylation activities were monitored during chromatography of human liver microsomal fractions. The two activities could be separated by chromatography, and proteins were purified to near-homogeneity that catalyzed either tolbutamide hydroxylation ($P\text{-}450_{TB}$) or (*S*)-mephenytoin 4′-hydroxylation ($P\text{-}450_{MP}$) but not both. Approximately 16 and 45% of the primary sequences of $P\text{-}450_{TB}$ and $P\text{-}450_{MP}$, respectively, were determined by analysis of the tryptic peptides. The sequences of the $P\text{-}450_{TB}$ peptides matched those predicted by the P-450 2C9 and 2C10 cDNAs exactly; the $P\text{-}450_{MP}$ peptides showed two mismatches (of 219 residues) with the P-450 2C10 sequence. Proteins with the P-450 2C10 and P-450 2C9 sequences were expressed in *Saccharomyces cerevisiae* grown under different nutritional conditions, and both were found to be proficient in the hydroxylation of tolbutamide but not (*S*)-mephenytoin. We conclude, on the basis of this and previous work, that 1) P-450s 2C8, 2C9, and 2C10 all catalyze the hydroxylation of tolbutamide and 2) the protein involved in polymorphic (*S*)-mephenytoin 4′-hydroxylation is closely related to but distinct from P-450 2C8, 2C9, and 2C10.

Approximately 3% of Caucasians and 20% of Orientals are deficient in the ability to catalyze the 4′-hydroxylation of the *S* enantiomer of the drug mephenytoin (1–4) (Fig. 1). Both *in vivo* and *in vitro* investigations indicate that the *R*-enantiomer is *N*-demethylated and not hydroxylated at the 4′-position; another enzyme is responsible for this activity. A trimodal polymorphism in the hydroxylation of the drug tolbutamide has been reported (5) but never confirmed. These hydroxylations are catalyzed by P-450 enzymes,[3] and efforts have been made to understand the phenomena at the molecular level. In 1985, we purified two proteins that were able to catalyze (*S*)-mephenytoin 4′-hydroxylation (7). Subsequent work with tolbutamide suggested that the same protein(s) might catalyze its hydroxylation, but discrepancies in the *in vivo* phenotypes were noted (8). Further studies indicated that sulfaphenazole is a competitive inhibitor of tolbutamide hydroxylation but mephenytoin is not (9), and sulfaphenazole was independently shown to be an efficient competitive inhibitor of tolbutamide hydroxylation but not (*S*)-mephenytoin 4′-hydroxylation (10). cDNAs termed P-450 2C8, 2C9, and 2C10 have been isolated and shown to be closely related (>80%) and the products of distinct genes (6, 11–14). A P-450 2C10 cDNA clone was expressed in a pAAH5 plasmid vector in *Saccharomyces cerevisiae*, and the resulting P-450 had activity towards tolbutamide but not mephenytoin (10). Yasumori *et al.* (15, 16) expressed a P-450 2C9 clone in a *S. cerevisiae* gal7 vector system; the protein had selective 4′-hydroxylation activity towards the *S*-enantiomer of mephenytoin, as seen in human liver (1), but the

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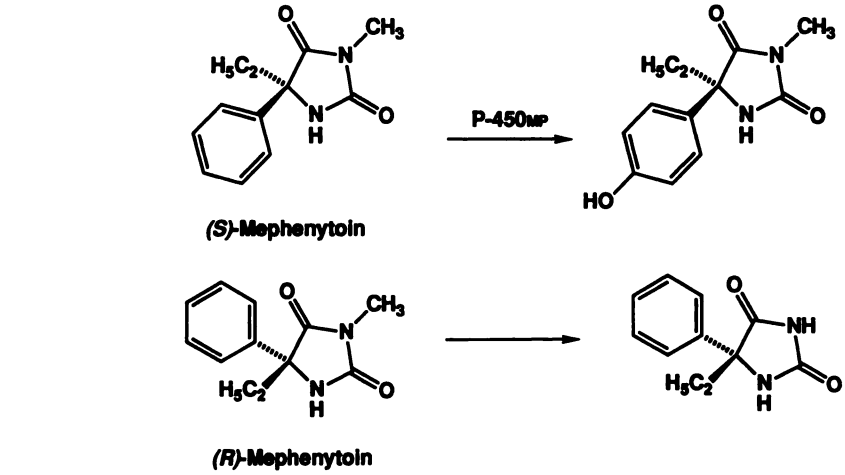
[3]For consideration of the nomenclature of P-450 2C and other P-450s, see Nebert *et al.* (6); in this work, the isolated proteins are designated $P\text{-}450_{MP}$ and $P\text{-}450_{TB}$. $P\text{-}450_{TB}$ is shown here to be P-450 2C9, by sequencing, but $P\text{-}450_{MP}$, postulated to be the polymorphic (*S*)-mephenytoin 4′-hydroxylase, cannot be assigned a complete sequence or a definitive classification (other than P-450 2C) at this time.

ABBREVIATIONS: P-450, liver microsomal cytochrome P-450; HPLC, high performance liquid chromatography; YPD (media), 2% (w/v) bactopeptone (Difco, Detroit, MI), 1% yeast extract (w/v, Difco), and 2% (w/v) glucose; YNBD (media), 0.67% yeast nitrogen base without amino acids (w/v, Difco) and 2% glucose (w/v).



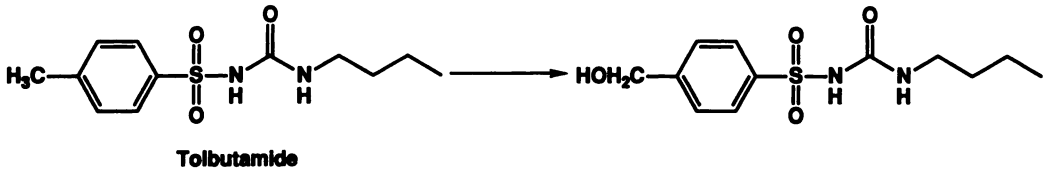


Fig. 1. Oxidations of mephenytoin and tolbutamide catalyzed by human P-450s.

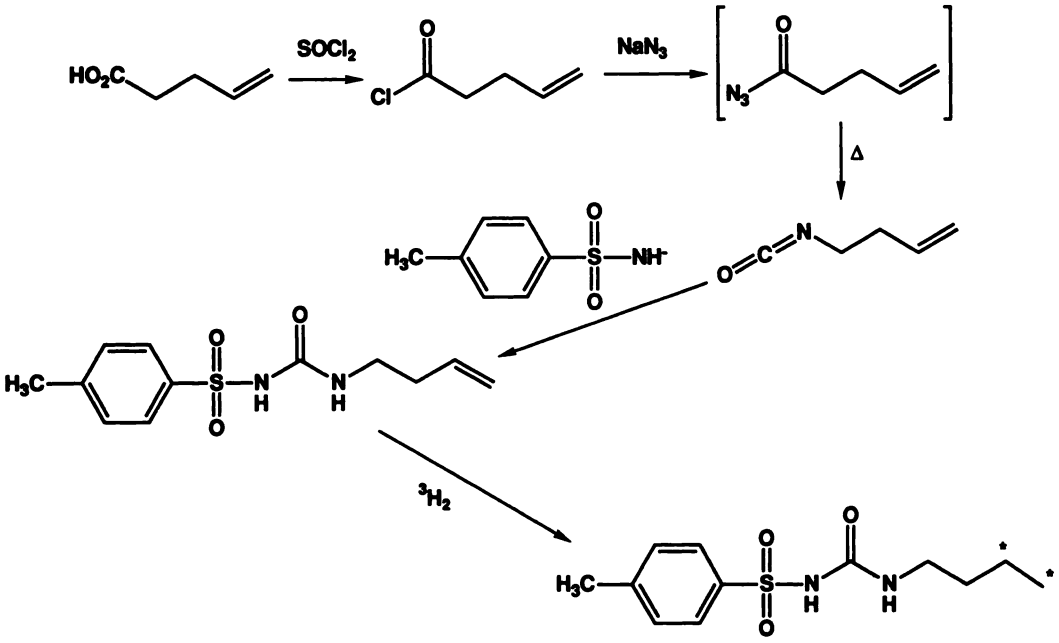


Fig. 2. Synthesis of [1,2-$^3H_2$]tolbutamide.

rate of activity was considerably lower than that seen in human liver microsomes and tolbutamide hydroxylation was not measured. Because P-450 2C9 differs from P-450 2C10 in only two residues of the protein-coding sequence, it is of interest to define the catalytic activities of these closely related proteins.

In this report, we compared the catalytic activities of the P450 2C9 and 2C10 proteins expressed in *S. cerevisiae* and also separated and purified proteins that selectively catalyze (*S*)-mephenytoin and tolbutamide hydroxylation activities from human liver microsomes. Comparison of the partial amino acid sequences of these proteins and those predicted by the available cDNA sequences indicates a high degree of similarity and suggests that (*S*)-mephenytoin 4′-hydroxylase activity may be the result of very limited sequence differences in the protein family.

Experimental Procedures

Chemicals. ICH_2CO_2H was recrystallized from hexane before use (in protein modification before sequence analysis). CF_3CO_2H was sequentially heated with and distilled from CrO_3 and alumina before use in HPLC. (*S*)-[*N*-*methyl*-^{14}C]Mephenytoin was prepared as described elsewhere (17); it was partitioned into CH_2Cl_2 and washed with 0.1 N NaOH to remove traces of residual nirvanol. The product was then crystallized from a mixture of $(CH_3)_2SO$ and H_2O (~1:1); radiochemical purity was >99.5% when analyzed using the analytical HPLC system (see below) (10), with no detectable radioactivity eluting in the region of the product.

[1,2-$^3H_2$]Tolbutamide was prepared in the following manner (Fig. 2). 1-Pentenoic acid (25.0 g, 0.25 mol) was mixed with 0.35 mol of $SOCl_2$ (41.6 g) and 0.025 mol of $(CH_3)_2NCHO$ (1.8 g), under a $CaCl_2$ drying tube (18, 19). The mixture was heated to reflux for 30 min and then

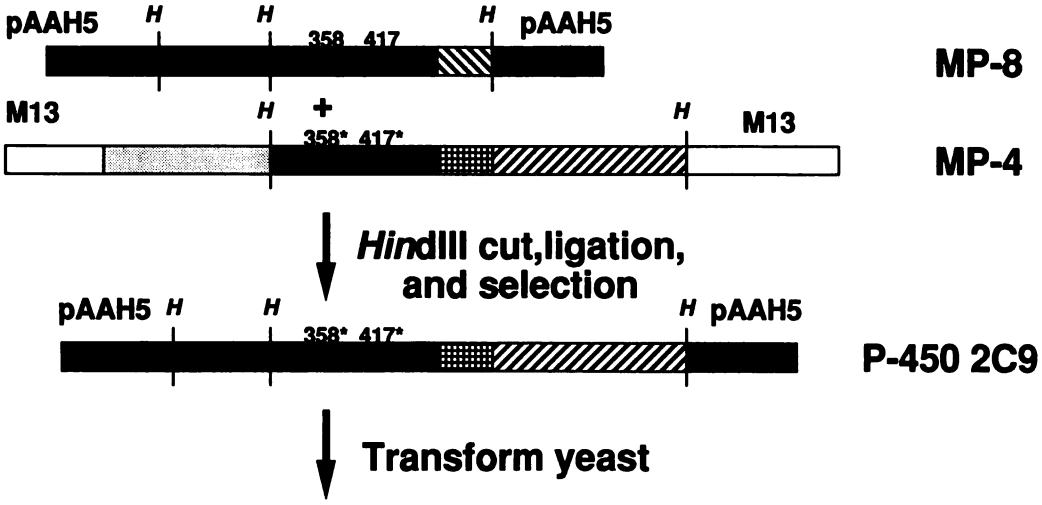


Fig. 3. Preparation of P-450 2C9 in pAAH5. The strategy is presented in detail in the text. ■, pAAH5 plasmid expression vector; ■, protein-coding region of cDNA clone MP-8; ▧, 3′ noncoding region of cDNA clone MP-8; □, bacteriophage M13; □, 5′ coding region of cDNA clone MP-4; ■, coding region of MP-4 (incomplete); ■, 3′ coding region of cDNA clone MP-4; ▨, 3′ noncoding region of cDNA clone MP-4. The codons that were altered (358 and 417) are indicated.

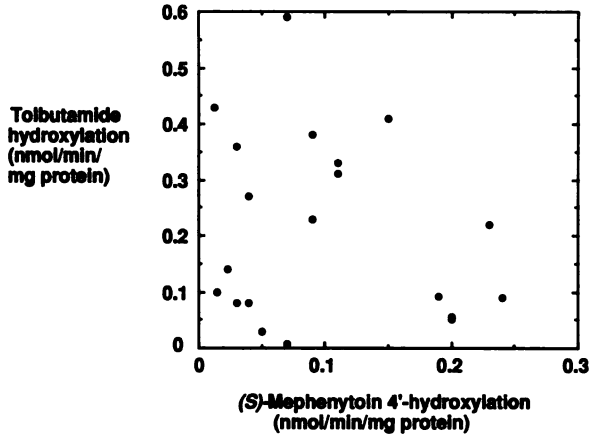


Fig. 4. Comparison of rates of (*S*)-mephenytoin 4′-hydroxylation and tolbutamide hydroxylation in 20 human liver microsomal samples. The apparent correlation coefficient (*r*) was −0.19 (linear regression analysis).

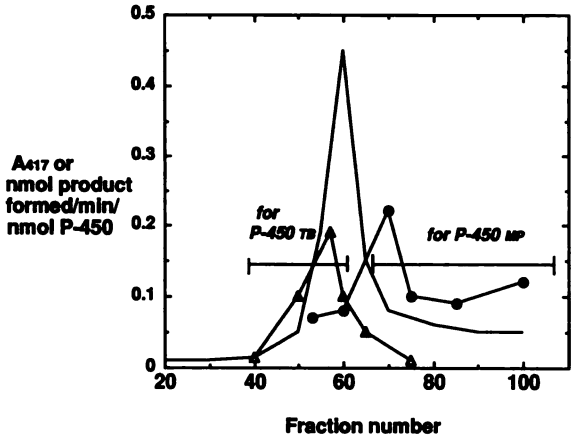


Fig. 5. Separation of human liver microsomal tolbutamide hydroxylation and (*S*)-mephenytoin 4′-hydroxylation activities by chromatography. Solubilized microsomes prepared from human liver sample HL 115 were applied to an *n*-octylamino-Sepharose 4B column, and the column was eluted with a stepwise gradient, as described in Experimental Procedures. Fractions were analyzed for A_{417} (——), tolbutamide hydroxylation activity (△), and (*S*)-mephenytoin 4′-hydroxylation activity (●).

TABLE 1

Catalytic activities of P-450 enzymes isolated from human liver microsomes

Form of P-450	Substrate	Catalytic activity	
		Liver HL 123	Liver HL 115
		nmol of product formed/ min/nmol of P-450	
Microsomes	Tolbutamide	0.09	0.05
	(*S*)-Mephenytoin	0.014	0.20
Purified $P\text{-}450_{TB}$	Tolbutamide	0.57	
	(*S*)-Mephenytoin	<0.01	
Purified $P\text{-}450_{MP}$	Tolbutamide		<0.01
	(*S*)-Mephenytoin		0.21
	(*R*)-Mephenytoin		<0.01

stirred overnight at room temperature. The product, 1-pentenoyl chloride, was distilled under aspirator vacuum (~18 mm of Hg). The fraction boiling between 37° and 47° (21.2 g, 71% yield) was used in the next step. An aqueous solution of NaN_3 (15.3 g in 50 ml) was stirred at 0°, and a solution of 20 g of 1-pentenoyl chloride (0.17 mol) in 50 ml of cold acetone was added dropwise, with the temperature being maintained at 10–15° (20). The mixture was then allowed to come to room temperature and was stirred for 1 hr. The upper phase was transferred to 170 ml of benzene and heated at 60–70° for 1 hr. The benzene was removed by distillation at atmospheric pressure, and then 3-butenyl isocyanate was recovered by further distillation (through a Vigareaux column). Fractions boiling in the ranges of 85–95° (10.5 g) and 105–115° (7.1 g) were collected (43% yield based on the latter fraction; only this was used in the subsequent step). *p*-Toluenesulfonamide (3.4 g, 0.020 mol) was stirred with 2.4 g of a 60% dispersion of NaH (0.060 mol) in mineral oil, in 100 ml of distilled (over $LiAlH_4$) tetrahydrofuran, at −10°. 3-Butenyl isothiocyanate (2.5 g, 0.026 mol) was added dropwise, with the temperature being maintained at −10°. The mixture was then allowed to come to room temperature and was stirred for 2 hr, after which it was quenched with 2.7 g of NH_4Cl (0.05 mol). The solution was made alkaline by the addition of 50 ml of 0.1 N NaOH, and the mixture was washed twice with CH_2Cl_2. The pH was lowered to ≤2 by the addition of HCl, and the product, *N*-[(3-butenylamino)carbamyl]-4-methylbenzene sulfonamide,[4] was extracted into CH_2Cl_2 (three times); the solution was dried with Na_2SO_4 and concentrated *in vacuo* to give 3.8 g (70%) of the product, which was crystallized from hot aqueous C_2H_5OH: m.p. 112–112.5°; λ_{max}, 228 (ϵ_{228}, 12.0 mM^{-1} cm^{-1}; in CH_3OH); ^1H NMR [C^2HCl_3, ppm relative to $(CH_3)_4Si$], δ 2.25 ($—CH_2—CH{=}CH_2$, 2 H, m), δ 2.46 ($—CH_3$, 3 H, s), δ 3.30 ($—NH—CH_2—$, 2 H, d of d), δ 5.11 ($=CH_2$, 2 H, d of d), δ 5.78 [$CH{=}CH_2$, 1 H, m, and $—NH—CH_2$, 1 H, s(b), superimposed], δ 6.58 [$—SO_2—NH—$, 1 H, s(b)], and δ 7.75 (aromatic protons, 4 H, apparent d of d); electron impact mass spectrum (relative abundance), *m/z* 269 (M + 1; 5), 268

[4]This compound was not found, in a search of Chemical Abstracts, to have been prepared previously.

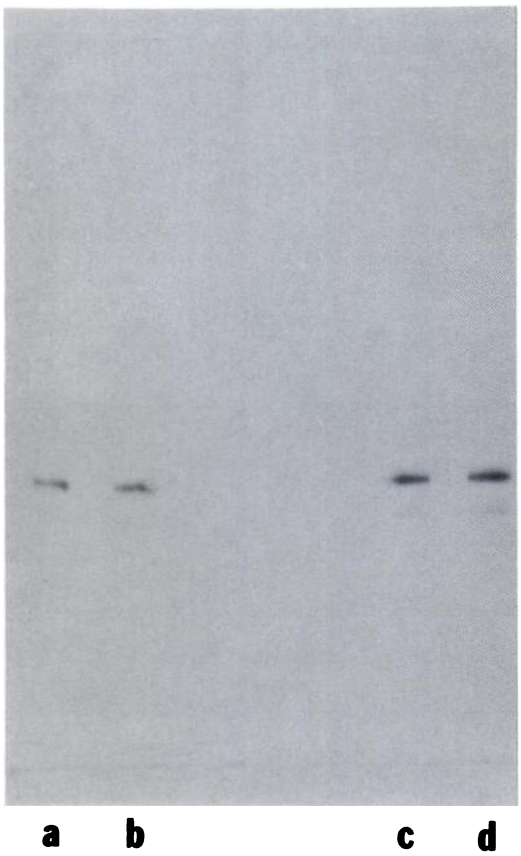


Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified (*S*)-mephenytoin 4′-hydroxylase preparations. The anode was at the *bottom* of the gel, and all lanes contained ~2 μg of protein. *Lanes a and b*, P-450$_{MP}$ purified from liver sample HL 115; *lanes c and d*, P-450$_{TB}$ purified from liver sample HL 123.

TABLE 2

Catalytic activities of microsomes prepared from *S. cerevisiae* expressing different P-450 2C cDNA sequences

cDNA	Residue		Medium	Catalytic activity		
	358	417		Tolbutamide	(*S*)-Mephenytoin	(*R*)-Mephenytoin
				nmol of product formed/min/nmol of P-450		
2C9	Tyr	Gly	YPD	3.3	<0.01	<0.01
2C9	Tyr	Gly	YNBD	2.2	<0.01	<0.01
2C10	Cys	Asp	YNBD	4.1	<0.01	<0.01

(M^+; 8), 227 (M − 41, loss of propene; 100), 155 (53), and 91 (toluyl; 100).

The material described above was used to prepare [^{3}H]tolbutamide by catalytic tritium reduction with H_2 (New England Nuclear, Boston, MA). The ^{3}H material was purified by preparative thin layer chromatography (200-μm silica gel G, $CHCl_3/CH_3OH$, 9:1, v/v) and HPLC (in the system used for analysis of oxidation products; (see below); the radiochemical purity (analyzed in the same system) was >99.5%, with no detectable radioactivity eluting in the region of the product. The specific radioactivity was 6 Ci/mmol, which was diluted to 2–6 mCi/mol in the assays.

Assays. Protein concentrations were estimated using a bicinchoninic acid procedure, as described by the supplier (Pierce Chemical Co., Rockford, IL). P-450 concentrations were estimated by Fe^{2+}-CO versus Fe^{2+} difference spectroscopy (21). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis utilized the basic procedure of Laemmli (22) (7.5% acrylamide); protein staining with silver was done according to the method of Wray *et al.* (23).

(*S*)-[*N-methyl*-^{14}C]Mephenytoin (11 mCi/mmol) and [1,2-$^3H_2$]tolbutamide (2–6 mCi/mol) were used in incubations where rates of hydroxylation were to be measured. The general incubation conditions are described elsewhere (8, 10, 17). When P-450 fractions were used in assays (from chromatography), the detergent concentration was lowered by shaking of an aliquot with a 20-fold excess of Amberlite XAD-2 beads (weight of beads/weight of Emulgen 911) for 2 hr before the assay (at 4°). Rabbit NADPH-P-450 reductase and L-α-dilauroyl-*sn*-glycero-3-phosphocholine were used with the P-450 (7). After incubation and acidification, the substrate and product were extracted into 4 volumes of CH_2Cl_2 and, after separation of the phases by centrifugation, the CH_2Cl_2 was removed under a stream of N_2. The residue was dissolved in 50–100 μl of CH_3CN, and an aliquot (20 μl) was injected onto an HPLC column (for elution conditions for 4′-hydroxymephenytoin and hydroxytolbutamide, see Ref. 10). The effluent was mixed with Flo-scint II liquid scintillation cocktail in a Radiomatic Flo-one counter (Radiomatic, Tampa, FL). In the radioactive tolbutamide hydroxylation assay, product formation was shown to be linear for up to 60 min and proportional to P-450 concentration in the ranges of 50–200 pmol of microsomal P-450 and 20–50 pmol of purified P-450. Hexobarbital 3′-hydroxylation was measured as described elsewhere (24).

Proteins were subjected to reductive alkylation with iodoacetic acid and digested with L-1-(tosylamido)-2-phenylethylchloromethylketone-treated trypsin (Worthington, Freehold, NJ), and peptides were isolated essentially as described elsewhere (12). The absorbances (A_{214}) of HPLC solvents were balanced before use by titration with distilled CF_3CO_2H (see above). Gradients of increasing CH_3CN in 0.1% aqueous CF_3CO_2H were used, first a broad gradient and then more shallow gradients for rechromatography of individual peptides (25). The amino acid sequences were determined by T. Porter in the Vanderbilt University facility, using automated Edman degradation, with an Applied Biosystems 470A sequenator; phenylthiohydantoins were analyzed online by HPLC. Yields of individual residues were estimated by integration of the chromatograms and comparison with external standards.

cDNA expression in yeast. P-450 2C9 and 2C10 differ in their coding sequences by only two amino acids, at positions 358 and 417 (12–14). P-450 2C10 cDNA has already been expressed in *S. cerevisiae* by Brian *et al.* (10), after addition of the 15 5′ nucleotides to MP-8 cDNA (11), insertion of the modified cDNA in the yeast expression vector pAAH5, and transformation of yeast with this plasmid. MP-4, a partial-length P-450 2C9 cDNA clone described by Ged *et al.* (12), is identical to 2C10 in its 3′ portion and was available in bacteriophage λgt11. It was inserted into M13mp18 phage (New England Biolabs, Beverly, MA) after digestion by *Eco*RI on the 5′ side and *Kpn*I on the 3′ side. This digestion liberated a fragment including all of the MP-4 (2C9) cDNA and about 1 kilobase of the λgt11 right arm on the 3′ end of MP-4 (Fig. 3). There is a *Hin*dIII restriction site present 855 base pairs 3′ to the initial ATG in both the P-450 2C9 and 2C10 cDNAs, and *Hin*dII is also a cloning site in the pAAH5 vector. After digestion with *Hin*dIII, this M13 fragment, pAAH5 yeast expression plasmid, and MP-8 (in M13mp18) were mixed and ligated overnight, using the protocol described by the supplier (New England Biolabs). *Escherichia coli* MC1060 cells were transformed with the ligation mixture, and the ampicillin-resistant colonies were screened with an oligonucleotide [no. 206 (12)] specific for the 5′ end of clone MP-8 (2C10); the positive colonies were then screened with a mixture of two oligonucleotides specific for the 3′ end of MP-4 (2C9). The positive clones were isolated and checked by restriction mapping (*Hin*dIII, *Bam*HI, and *Sph*I) for correct insertion of the hybrid cDNA, namely the 5′ end of MP-8 and the 3′ end of MP-4. One clone had the inserts in the proper order and orientation. This plasmid was used for transformation of *S. cerevisiae* strain AH22, in parallel with transformations with the pAAH5 plasmid and pAAH5 containing MP-8 cDNA (2C10). Isolated colonies were selected and grown either on rich (YPD) or minimal (YNBD) medium (10). The plasmids were rescued from yeast and checked by restriction mapping. Yeast microsomes were prepared as described (10) and were used to measure P-450 content (21), enzymatic activities, and P-450

TABLE 3

Yields of residues in amino acid sequencing of peptides

Residue	P-450$_{TB}$ peptides												P-450$_{MP}$ peptides																					
	40–47		109–116		242–247		276–298		384–399		400–417		1–11		49–52		85–97		98–104		109–116		120–125		122–125		133–137		140–144		145–150			
	AA[a]	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol		
1	G	150	G	220	S	141	H	69	G	181	E	155	M	21	D	171	E	611	G	205	G	659	W	85	E	186	N (or G)[b]		S	147	V	375		
2	N	139	F	153	Y	139	N	89	T	103	F	160	D	32	I	140	A	283	I	168	F	414	K	87	I	180	F	27	I	239	Q	291		
3	I	233	G	168	I	145	Q	129	T	117	P	170	—		S (or C)		L	184	F	152	G	254	E	137	R	40	G	44	E	191	E	288		
4	L	276	I	139	L	86	P	107	I	114	N	127	L	15	K	36	I	186	P	174	I	246	I	91	R	30	M	21	D	143	E	298		
5	Q	220	V	137	E	82	S	63	L	104	P	147	V	11			D	210	L	135	V	210					G	40	R	33	A	267		
6	I	90	F	130	K	64	E	92	I	101	E	97	V	13			L	149	A	94	F	204									R	30		
7	G	100	S	55			F	81	S	84	M	184	L	17			G	156	E	99	S	84												
8	I	31	N	110			T	60	L	75	F	128	V	12			E	261			N	21												
9							I	61	T	53	D	95	L	19			E	259																
10							E	47	S	56	P	97	—				F	101																
11							S	42	V	25	—		L	18			S	96																
12							L	44	L	39	—						G	80																
13							E	40	H	14	F	72					R	26																
14							N	28	D	25	L	74																						
15							T	50	N	24	D	58																						
16							A	30	K	16	E	43																						
17							V	19			G	54																						
18							D	26			D	ND[e]																						
19							L	22																										
20							F	20																										
21							G	19																										
22							A	38																										
23							G	21																										

Residue	P-450$_{MP}$ peptides																																	
	161–181		186–190		187–199		201–206		236–239		236–241		242–247		248–264		276–289		323–329		343–357		358–374		378–382		400–416		460–465		466–475			
	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol	AA	pmol		
1	A	64	R	39	F	96	L	185	N	82	—[c]		S	79	V	212	G	145	V	913	S	301	Y	115	N	143	E	74	S	30	N	246		
2	S	17	F	41	D	85	N	282	V	65	V	15	Y	206	K	70	N	110	Q	599	H	67	I	60	Y	138	F	62	L	12	L	131		
3	P	44	D	42	Y	84	E	173	A	33	A	9	I	132	E	93	Q	101	E	620	M	214	D	74	L	61	P	45	V	8	D	222		
4	C	NC[d]	Y	32	—		N	175	F	10	F	6	L	116	H	25	P	84	E	684	P	119	L	46	I	33	N	74	D	15	T	106		
5	D	45	K	7	D	67	I	88			M	5	E	81	Q	88	S	37	I	573	Y	169	L	45	P	31	P	45	P	11	T	111		
6	P	34			Q	68	K	35			K	2	K	18	E	60	E	72	K	452	T	146	P	81			E	63	K	2	P	79		
7	T	19			Q	54									—		F	55	K	111	D	159	T	32			M	71			V	60		
8	F	24			F	35									M	36	T	53			A	115	S (or W)	15 (8)			F	45			V	69		
9	I	26			L	33									D	44	I	44			V	43	L	16			D	71			N	9		
10	L	20			N	35									M	21	E	55			V	61	P	52			P	30			G	15		
11	G	28			L	26									N	20	S	26			H	31	—				H	7						
12	C	NC			M	24									N	25	L	20			E	57	A	21			H	13						
13	A	17			E	20									P	12	—				V	28	V	11			F	16						
14	P	17													Q	11	N	30			Q	36	T	11			L	11						
15	C	NC													D	9					R	22	C (or S)				D	30						
16	N	14													F	5							D	19			E	15						
17	V	5													I	3							I	8			G	16						
18	I	10																																
19	C	NC																																
20	—																																	
21	I	4																																
22																																		
23																																		

[a] Amino acid residue. [b] Two residues were recovered. [c] No residue was identified at the indicated cycle. [d] NC, yields of C (recovered as carboxymethylcysteine) were not calculated. [e] ND, yield not determined.

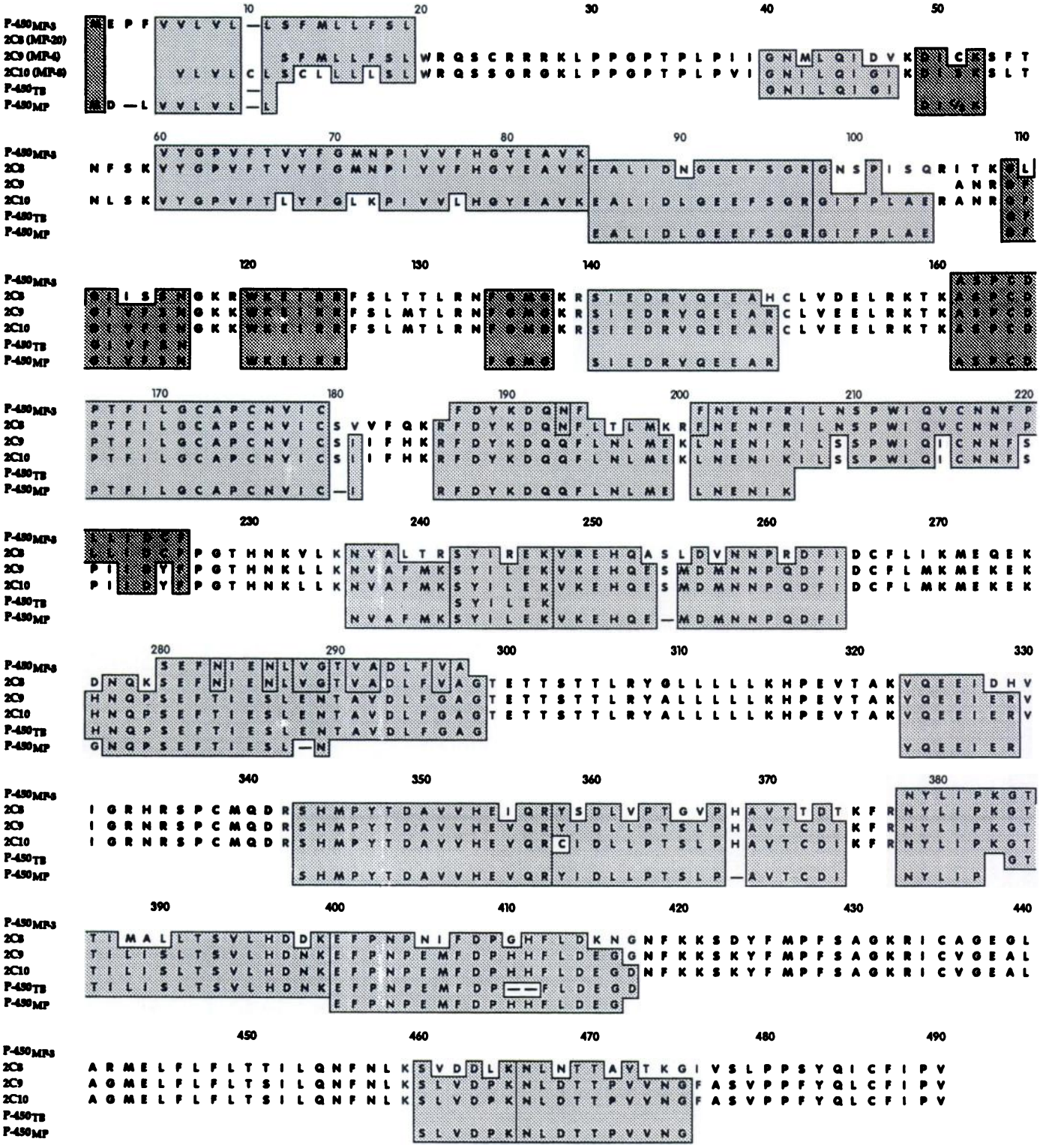


Fig. 7. Comparison of amino acid sequences predicted by P-450 2C8, P-450 2C9, and P-450 2C10 cDNA sequences and alignment with amino acid sequences of peptides isolated from $P\text{-}450_{MP\text{-}3}$ (11) and $P\text{-}450_{TB}$ and $P\text{-}450_{MP}$ (this work). See Table 3 for yields of residues from each peptide. The P-450 2C8, 2C9, and 2C10 sequences are those of the respective cDNA clones MP-20, MP-4, and MP-8 previously reported by this laboratory (11), without addition of missing segments. *Shading* is only shown when amino acid sequences are being compared.

2C content (by immunoblotting). All the microsomes prepared from yeasts transformed either with P-450 2C9 (MP-4 derivative) or P-450 2C10 (MP-8 derivative) cDNA contained spectrally measurable P-450 (levels were similar), whereas P-450 content was below the limit of detection in microsomes prepared from the yeast transformed with pAAH5 (see Ref. 10). Immunoblots probed with anti-$P\text{-}450_{MP}$ (7) showed that both yeasts transformed with plasmids containing P-450 2C9 and 2C10 cDNAs synthesized a protein that was recognized by the antibody and migrated with purified $P\text{-}450_{MP}$.

Purification of human P-450s. Twenty human liver microsomal preparations were screened for (*S*)-mephenytoin 4′-hydroxylation and tolbutamide methyl hydroxylation activities (Fig. 4). Sample HL 115

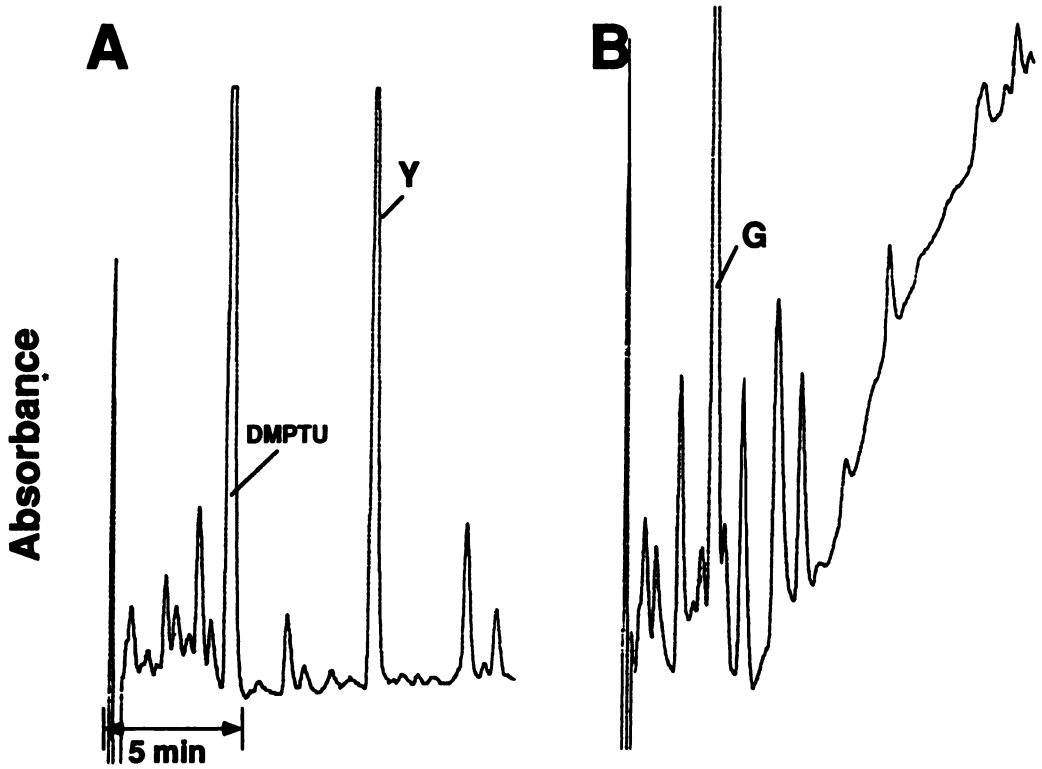


Fig. 8. HPLC of phenylthiohydantoins recovered from selected cycles of Edman degradation of peptides isolated from purified P-450$_{MP}$ (from liver sample HL 115). A, position 358 (*DMPTU, N,N*-dimethylphenylthiourea); B, position 276. Peaks were identified by comparison of retention times with standards.

was selected for use because it had high (*S*)-mephenytoin 4′-hydroxylation and relatively low tolbutamide methyl hydroxylation activity (Table 1).

P-450$_{MP}$ was purified according to the method of Shimada *et al.* (7), with slight modification. Briefly, P-450s were solubilized with sodium cholate, loaded onto 2.5- × 48-cm *n*-octylamino-Sepharose 4B columns, and eluted with 10 mM potassium phosphate buffer (pH 7.4) containing 0.4% (w/v) sodium cholate, 1 mM EDTA, 20% (v/v) glycerol, and 0.06% (w/v) Emulgen 911 (eluate I); further elution was performed with the same buffer containing 100 mM potassium phosphate buffer (pH 7.4) and 0.2% (w/v) Emulgen 911 (eluate II). Tolbutamide hydroxylase was found mainly in the earlier fractions (fractions 40–64), whereas (*S*)-mephenytoin 4′-hydroxylation activity was eluted in the later fractions (fractions 65–120) of eluate I (Fig. 5). Those fractions having relatively high (*S*)-mephenytoin 4′-hydroxylation activity were pooled, dialyzed, and applied to a 2.5- × 9-cm column of hydroxylapatite (high resolution grade; Calbiochem, San Diego, CA). The hydroxylapatite column was eluted sequentially with 40, 90, and 180 mM potassium phosphate buffers (pH 7.25) containing 0.2 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911. (*S*)-Mephenytoin 4′-hydroxylation activity was found primarily in the 180 mM phosphate fractions, and no detectable tolbutamide hydroxylation activity was present. These fractions were pooled, dialyzed extensively to reduce the phosphate concentration, and applied to a 1.6- × 10-cm Whatman DE-52 DEAE-cellulose column (Whatman, Clifton, NJ), which had been equilibrated with 5 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA, 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911. (*S*)-Mephenytoin 4′-hydroxylation activity was eluted only in the void volume. The pH of this fraction was adjusted to 6.75 with 1 N CH_3CO_2H, and the material was applied to a 1.6- × 6-cm Whatman CM-52 CM-cellulose column, which had been previously equilibrated with 7.5 mM potassium phosphate buffer (pH 6.75) containing 0.1 mM EDTA, 0.2 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% (w/v) Emulgen 911. The column was washed with 50 ml of the equilibration buffer, and P-450$_{MP}$ was eluted with a 100-ml linear gradient of 0–150 mM NaCl in the same buffer. The protein that eluted at an NaCl concentration of ~100 mM contained mephenytoin 4′-hydroxylation activity and was almost homogeneous, with the exception of one contaminating protein. These fractions were dialyzed overnight against 5 mM potassium phosphate buffer (pH 6.5) containing 0.15 mM EDTA, 0.2 mM dithiothreitol, and 20% (v/v) glycerol and were applied to a 1.6- × 4.5-cm CM-Sepharose CL-6B column (Pharmacia, Piscataway, NJ), which was equilibrated with the same buffer at room temperature. The column was washed with 40 ml of the equilibration buffer and eluted with a 100-ml linear gradient of 0–200 mM NaCl in the same buffer (at room temperature). The protein fraction that eluted in the 100–120 mM region of the gradient was electrophoretically homogeneous (Fig. 6) and contained (*S*)-mephenytoin 4′-hydroxylation activity but no tolbutamide hydroxylation activity. Those fractions containing P-450$_{MP}$ were pooled, and Emulgen 911 was removed from the purified preparation by sequential adsorption of protein on a small column (1.2 × 1.5 cm) of hydroxylapatite, extensive washing with CM-52 equilibration buffer devoid of detergent (with monitoring of A_{280}), and elution with 0.5 M potassium phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol and 20% (v/v) glycerol. The final preparation was dialyzed overnight against 40 volumes of 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol and was stored at −20°.

The overall yield, based upon total microsomal P-450, was about 0.4%; it is not practical to estimate the recovery of the (*S*)-mephenytoin 4′-hydroxylation activity because of difficulty in comparing the results of microsomal assays with those using the reconstituted system.

The same general procedure was used with fractions 65–120 eluted from the *n*-octylamino-Sepharose 4B column (Fig. 5), to purify an enzyme that had tolbutamide hydroxylation activity but was devoid of (*S*)-mephenytoin 4′-hydroxylation activity (11).

Results

Development of radiochromatographic assay conditions. Previous work on the expression of P-450 2C cDNAs in

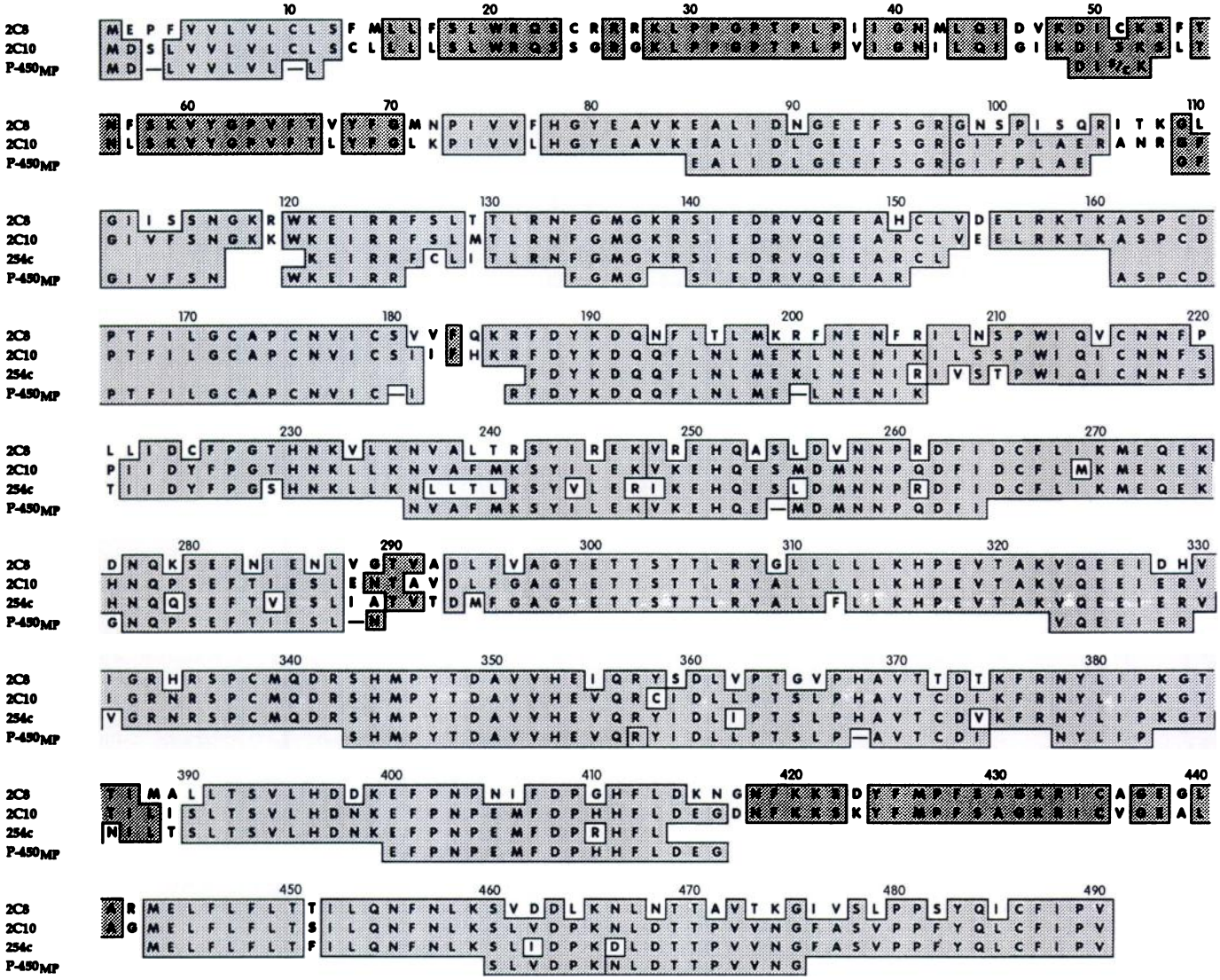


Fig. 9. Comparison of amino acid sequences predicted by P-450 2C8, 2C10, and 254c (25) sequences with that of P-450$_{MP}$ determined by Edman degradation in this work.

TABLE 4

Putative substrates for human P-450 2C proteins

Compound	Enzyme	Reference
Tolbutamide	2C8, 2C9, 2C10	10, 29
Hexobarbital	2C10 (P-450$_{MP}$)	10, 15, 23, 29
(*S*)-Mephenytoin	P-450$_{MP}$	This work
Tienilic acid	?	30
Retinol	?	31
Retinoic acid	?	31
Diazepam	?	32
Nirvanol	P-450$_{MP}$	7, 33
Ethotoin	P-450$_{MP}$?	26
Mephobarbital	P-450$_{MP}$	26, 33, 34
Methsuximide	P-450$_{MP}$?	26
Phensuximide	P-450$_{MP}$?	26
Phenytoin	2C8 ?	35

yeast suggested that the P-450 enzymes involved in the hydroxylation of (*S*)-mephenytoin and tolbutamide are distinct but closely related. Sensitive radiometric assays for both hydroxylation activities were deemed necessary to monitor separation and purification. [1,2-$^3H_2$]Tolbutamide was prepared using the synthetic scheme described (Fig. 2) and was used in radio-HPLC assays. The assay could be done with ≤10 pmol of microsomal P-450. These assays were both routinely used to monitor catalytic activity during purification.

Catalytic activities of P-450 2C9 and P-450 2C10 expressed in yeast. Previous work in this laboratory indicated that microsomes prepared from *S. cerevisiae* expressing P-450 2C10 were highly active in catalyzing tolbutamide hydroxylation but not (*S*)-mephenytoin 4′-hydroxylation (10). Yasumori *et al.* (15, 16) have reported very low rates of mephenytoin hydroxylation in yeast expressing P-450 2C9, a cDNA that differs from P-450 2C10 in only two residues (Tyr instead of Cys at 358 and Gly instead of Asp at 417). We changed both residues in P-450 2C10 by moving an appropriate 2C9 cDNA segment, so that an identical protein (P-450 2C9) is expressed in the yeast (Fig. 3). The two proteins had very similar catalytic activities, i.e., both were excellent tolbutamide hydroxylases but poor (*S*)-mephenytoin 4′-hydroxylases (Table 2). The substitution of a minimal medium for rich medium had little effect

on the level of P-450 expression or the catalytic activity of either protein (Table 2). In other experiments, which are not presented here, similar rates of 3′-hydroxylation of (±)-hexobarbital were measured for the different yeast preparations (10).

Purification of P-450$_{MP}$. In previous analyses, some *in vitro* correlation was seen between rates of hydroxylation of (*S*)-mephenytoin and of tolbutamide, but no *in vivo* correlation was noted (8). A larger group of human liver microsomal samples was analyzed, and no evidence for correlation was found (Fig. 4).

Two liver samples were selected from the group shown in Fig. 4. One (code HL 115) had relatively high (*S*)-mephenytoin 4′-hydroxylation activity and low tolbutamide hydroxylation activity. The other (code HL 123) had relatively low (*S*)-mephenytoin 4′-hydroxylation activity and high tolbutamide hydroxylation activity (Table 1). A number of chromatography systems were carefully examined, to determine which would be most useful in the separation of the two catalytic activities, measured with the sensitive radiochromatographic assays. Of the chromatography systems considered, the one that best separated the two activities from each other was the *n*-octylamino-Sepharose 4B column used in the first step (Fig. 5). Separation could be obtained using either isocratic elution (Fig. 5) or a linear gradient in which the concentrations of both potassium phosphate and Emulgen 913 were increased (results not shown). The indicated fractions (Fig. 5) were pooled and used in similar steps (see Experimental Procedures) to purify two proteins to apparent homogeneity (Fig. 6); these are denoted P-450$_{MP}$ and P-450$_{TB}$ (the P-450$_{TB}$ preparation contains a minor impurity).

The purified preparations were analyzed for catalytic activities (Table 2). P-450$_{TB}$ had tolbutamide hydroxylase activity but very little (*S*)-mephenytoin 4′-hydroxylation activity. P-450$_{MP}$ had no catalytic activity towards tolbutamide but catalyzed the 4′-hydroxylation of (*S*)-mephenytoin at a rate similar to that seen in the liver microsomal sample from which it was isolated. The entire purification scheme was carried out three times, and similar catalytic properties of the P-450$_{MP}$ preparations were observed.

Amino acid sequences of peptides. The P-450$_{TB}$ and P-450$_{MP}$ preparations were treated to block cysteine groups and were digested with trypsin to prepare peptides. Some of the isolated peptides were used for determination of amino acid sequences, and repetitive yields are reported in Table 3. All of the residues in the P-450$_{TB}$ protein match those predicted by the P-450 2C9 and 2C10 sequences (Fig. 7). (In addition, the sequencing with the intact protein showed the expected residues for P-450 2C10 at positions 1–9 and 10–12, but it was not possible to calculate yields due to an inadvertant error.) The match between the so-called "P-450$_{MP-3}$" protein previously isolated (12) and the predicted P-450 2C8 sequence is also shown (no mismatches among 120 residues). P-4502C8 protein was also found to have low tolbutamide hydroxylation activity (0.13 nmol of product formed/min/nmol of P-450) but no (*S*)-mephenytoin 4′-hydroxylase activity (<0.0005 nmol of product formed/min/nmol of P-450) (12).

The P-450$_{MP}$ protein isolated in this work is very similar to the sequences predicted from the cDNA clones. Two differences (of 219 residues) have been noted to date (Fig. 8). However, one is at position 358 (Tyr instead of Cys), which does not appear to be sufficient to give activity to the protein (Table 1). The other difference involves substitution of a Gly for His-276.

Recently, Romkes *et al.* (26) reported a partial sequence of a closely related cDNA clone ("254c") isolated from a human liver cDNA library. That sequence is ~85% identical to those of P-450s 2C8, 2C9, and 2C10. However, where clear reading frames exist, the predicted protein is less similar to the peptides isolated from P-450$_{MP}$ than are P-450 2C9 and P-450 2C10 (Fig. 9).

Discussion

Highly sensitive methods for the assay of catalytic activity were developed and used to monitor the purification of the human P-450 enzymes involved in (*S*)-mephenytoin and tolbutamide hydroxylation. The enzymes were resolved in the first step of the purification, arguing that the bulk of each activity is catalyzed by a different enzyme(s). The (*S*)-mephenytoin 4′-hydroxylation activity of the purified P-450$_{MP}$ protein was about 0.2 nmol of product formed/min/nmol of P-450, a value similar to those of the microsomal sample from which the protein was derived (Table 1) and of some earlier preparations of P-450$_{MP}$ (which had not been analyzed for tolbutamide hydroxylation activity) (7). It is clear from assays monitored by radio-thin layer chromatography (17), HPLC with UV detection (7), radio-HPLC (10), and combined gas chromatography/mass spectrometry using deuterated internal standards (27) that some of the faster rates of (*S*)-mephenytoin 4′-hydroxylation in the human liver microsomal samples are in the range of 0.2–0.5 nmol of product formed/min/nmol of P-450. Therefore, one might expect the catalytic activity of the purified P-450$_{MP}$ preparations to be higher than 0.2 nmol of product formed/min/mg of P-450, but it is possible that reconstitution conditions are not optimal (the addition of cytochrome b_5 to the enzyme had a stimulatory, but not dramatic, effect in these experiments). A precedent for loss of catalytic activity upon purification exists in the P-450 3A4 enzyme (28). However, the rate of tolbutamide hydroxylation seen with the purified P-450$_{TB}$ preparation was considerably higher than that in the particular microsomal preparation from which the protein was derived (Table 1); in the yeast expression systems, considerably higher rates are seen (Table 2).

The P-450 2C9 protein expressed in a similar yeast expression system by Yasumori *et al.* (14, 15) has a sequence identical to that of the P-450 2C9 protein made here. Yasumori *et al.* (16) have reported that their protein is stereoselective for (*S*)-mephenytoin (consistent with expectations from *in vivo* and *in vitro* human studies), but the rate of product formation, even in the presence of added reductase, was still only ~5% of that seen under a variety of assay conditions for human liver microsomes in several laboratories (10, 27, 29). These rates (15) are near the limit of detection in some of our own assays (Tables 1 and 2; Fig. 4) and, because the tolbutamide hydroxylation rates measured with these proteins are considerably higher than those in human liver microsomes, it does not appear that P-450 2C9 should be considered the true (*S*)-mephenytoin 4′-hydroxylase. Recently, Relling *et al.* (30) expressed P-450 2C8 and a derivative of P-450 2C9 in a mammalian cell system and also concluded that these enzymes are tolbutamide hydroxylases and not (*S*)-mephenytoin 4′-hydroxylases. It should be pointed out that studies with oligonucleotide probes prepared to specific sequences in the 3′ noncoding region argue that P-

450 2C8, P-450 2C9, and P-450 2C10 (or nearly matching sequences) can be concurrently expressed in a single human liver, along with the (*S*)-mephenytoin 4′-hydroxylase (12).

The partial cDNA clone of Romkes *et al.* (26) was considered as a new prospective candidate for the polymorphic (*S*)-mephenytoin 4′-hydroxylase. Although this possibility cannot be dismissed, alignment of partial sequences (Fig. 9) indicates that the amino acid sequence associated with the peptides isolated from the catalytically active P-450$_{MP}$ protein is 75% identical to P-450 2C8, 87% identical to the protein coded for by the 254c clone (26), and >98% identical to P-450 2C10 and P-450 2C9. Although these analyses can be deceptive, it would appear that the true polymorphic (*S*)-mephenytoin 4′-hydroxylase is most closely related to P-450 2C9 and P-450 2C10, of the sequences available at this time.

Several lines of evidence indicate that the human P-450 2C enzymes are involved in the oxidation of a number of drugs and endogenous compounds (Table 4). What is not clear in most cases is exactly which of the proteins can catalyze each reaction. In some instances, it is clear that several enzymes probably participate. Another point to be made is that K_m values have not been measured in all these situations, particularly with gene products expressed in artificial systems. Obviously, an enzyme with a low K_m should be more important at the substrate concentrations encountered *in vivo*. However, the K_m values reported for (*S*)-mephenytoin 4′-hydroxylation in human liver microsomes are relatively high (7, 10, 26), and the concentration of (*S*)-mephenytoin used here (0.5 mM) is only a few fold higher than the K_m observed in microsomes. Thus, we would not appear to be selecting against a low-K_m form that is functionally significant.

What, then, is the true (*S*)-mephenytoin 4′-hydroxylase? These studies would argue that the sequence of the P-450$_{MP}$ protein isolated here should describe this entity. However, it cannot be stated that this is the major (*S*)-mephenytoin 4′-hydroxylase without the addition of caveats. The significance of the few differences seen in the primary sequence is currently unclear, particularly because the cDNA library used to derive the DNA sequences was prepared from a different individual than was the catalytically active protein (however, the agreement of the nucleotide and amino acid sequences is excellent). An alternative possibility that cannot be dismissed is that a posttranslational modification of P-450 2C9 or P-450 2C10 occurs in hepatocytes, but not yeast or cultured cells, and alters the catalytic specificity of the protein. Further, it is not necessarily imperative that the major (*S*)-mephenytoin 4′-hydroxylase should be devoid of tolbutamide hydroxylation activity, but the results shown in Figs. 4 and 5 would argue that one should expect the tolbutamide hydroxylation rate to be considerably less than that measured for (*S*)-mephenytoin 4′-hydroxylation, and the existence of high catalytic activities towards (*S*)-mephenytoin and tolbutamide in a single protein would not be expected. There are several potential approaches for isolating the cDNA related to polymorphic (*S*)-mephenytoin 4′-hydroxylation at this point. One involves the expression of any related P-450 2C clones that can be isolated from a cDNA library (26). Another potential approach, which has not been applied, involves differential or subtractive screening. Both of these methods are theoretically possible but are difficult if only very small differences exist in the coding regions (we were unsuccessful in defining differences in genomic restriction maps of extensive and slow metabolizers, using a panel of more than 30 restriction endonucleases) (12). Our own strategy at this point involves further analysis of the amino acid sequence of the catalytically active P-450$_{MP}$, with a view to defining differences that will generate unambiguous oligonucleotide probes.

Author's note: Very recently Romkes et al [Romkes, M., M. B. Faletto, J. A. Blaisdell, J. L. Raucy, and J. A. Goldstein. *Biochemistry* **30**:3247–3255 (1991)] have published the sequences of human P-450 2C17, 2C18, and 2C19 cDNA clones (the 254c sequence cited here is part of P-4520 C17). When the P-450 2C9, 2C18, and 2C19 cDNA clones were expressed in COS-1 cells only P-450 2C18 showed (±) mephenytion 4′-hydroxylation activity above the endogenous level in the cells. A role of P-450 2C18 as a major (*S*)-mephenytoin 4′-hydroxylase is the subject of further investigation.

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