THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 2, Issue of January 15, pp. 909-921, 1986
Printed in U.S.A.

Human Liver Microsomal Cytochrome P-450 Mephenytoin 4-Hydroxylase, a Prototype of Genetic Polymorphism in Oxidative Drug Metabolism

PURIFICATION AND CHARACTERIZATION OF TWO SIMILAR FORMS INVOLVED IN THE REACTION*

(Received for publication, April 29, 1985)

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Two forms of cytochrome P-450 (P-450), designated P-450$_{MP-1}$ and P-450$_{MP-2}$, were purified to electrophoretic homogeneity from human liver microsomes on the basis of mephenytoin 4-hydroxylase activity. Purified P-450$_{MP-1}$ and P-450$_{MP-2}$ contained 12–17 nmol of P-450/mg of protein and had apparent monomeric molecular weights of 48,000 and 50,000, respectively. P-450$_{MP-1}$ and P-450$_{MP-2}$ were found to be very similar proteins as judged by chromatographic behavior on *n*-octylamino-Sepharose 4B, hydroxylapatite, and DEAE- and CM-cellulose columns, spectral properties, amino acid composition, peptide mapping, double immunodiffusion analysis, immunoinhibition, and N-terminal amino acid sequences. *In vitro* translation of liver RNA yielded polypeptides migrating with P-450$_{MP-1}$ *or* P-450$_{MP-2}$, depending upon which form was in each sample, indicating that the two P-450s are translated from different mRNAs.

When reconstituted with NADPH-cytochrome-P-450 reductase and L-α-dilauroyl-*sn*-glyceryo-3-phosphocholine, P-450$_{MP-1}$ and P-450$_{MP-2}$ gave apparently higher turnover numbers for mephenytoin 4-hydroxylation than did the P-450 in the microsomes. The addition of purified rat or human cytochrome b_5 to the reconstituted system caused a significant increase in the hydroxylation activity; the maximum stimulation was obtained when the molar ratio of cytochrome b_5 to P-450 was 3-fold. Rabbit anti-human cytochrome b_5 inhibited NADH-cytochrome-*c* reductase and *S*-mephenytoin 4-hydroxylase activities in human liver microsomes. In the presence of cytochrome b_5, the K_m value for *S*-mephenytoin was 1.25 mM with all five purified cytochrome P-450s preparations, and V_{max} values were 0.8–1.25 nmol of 4-hydroxy product formed per min/nmol of P-450. P-450$_{MP}$ is a relatively selective P-450 form that metabolizes substituted hydantoins well. Reactions catalyzed by purified P-450$_{MP-1}$ and P-450$_{MP-2}$ preparations and inhibited by anti-P-450$_{MP}$ in human liver microsomes include *S*-mephenytoin 4-hydroxylation, *S*-nirvanol 4-hydroxylation, *S*-mephenytoin *N*-demethylation, and diphenylhydantoin 4-hydroxylation. Thus, at least two very similar forms of human P-450 are involved in *S*-mephenytoin 4-hydroxylation, an activity which shows genetic polymorphism.

Cytochrome P-450 enzymes catalyze the monooxygenation of a wide variety of chemicals, including drugs, carcinogens, pesticides, pollutants, and other xenobiotics as well as endogenous steroids, fatty acids, and prostaglandins (Conney, 1967; Sato and Omura, 1978; Wislocki *et al.*, 1980). In recent years, numerous biochemical studies have led to the purification of many of the isozymes from experimental animals, determination of substrate specificities, the development of immunochemical techniques for quantitative estimation of individual isozymes, determination of primary sequences, and the application of recombinant DNA methods to questions concerning the regulation of these enzymes (Imai and Sato, 1974; Haugen and Coon, 1976; Norman *et al.*, 1978; Ryan *et al.*, 1979; Guengerich, 1979; Guengerich *et al.*, 1982b; Fujii-Kuriyama *et al.*, 1982; Cheng *et al.*, 1984; Yabusaki *et al.*, 1984; Kimura *et al.*, 1984).

Humans, of course, also contain P-450s.[1] Individual forms have been isolated and purified, and biochemical evidence supports the existence of isozymes (Wang *et al.*, 1980, 1983). However, little is known about the similarity of individual forms in different people and the substrate specificities of these P-450s. One of the more interesting aspects of the P-450s in humans relates to the polymorphic variation in the activities of certain of these enzymes toward the oxidation of certain drugs (Küpfer and Preisig, 1983; Mahgoub *et al.*, 1977; Scott and Poffenbarger, 1979; Küpfer *et al.*, 1984). In several cases, this polymorphism has a strong genetic component. Debrisoquine 4-hydroxylation has probably been the most

* This research was supported in part by Grants CA 30907 and ES 00267 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: P-450, liver microsomal cytochrome P-450; $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G fraction (of sera); PTH, phenylthiohydantoin; P-450$_{MP-1}$ and P-450$_{MP-2}$, the respective 48,000- and 50,000-dalton forms of the human *S*-mephenytoin 4-hydroxylase discussed in this report; P-450$_{DB}$, the human liver debrisoquine 4-hydroxylase purified elsewhere (Distlerath *et al.*, 1985); P-450 $_{PA}$, the human liver phenacetin *O*-deethylase purified elsewhere (Distlerath *et al.*, 1985). Human liver samples are denoted "HL" with a code number for each individual person. The properties of the rat P-450s designated P-450$_{PCN-E}$ and P-450$_{UT-I}$ have been described elsewhere (Guengerich *et al.*, 1982a; Waxman *et al.*, 1985). Other preparations which appear to correspond to P-450$_{PCN-E}$ (P-450$_{PB/PCN-E}$) include those designated "P-450$_{PCN}$" (Elshourbagy and Guzelian, 1980) and "PB-2a" (Waxman *et al.*, 1985). Other preparations which appear to correspond to P-450$_{UT-I}$ include "female-specific P-450" (Kamataki *et al.*, 1983), "P-450i" (Ryan *et al.*, 1984) and "P-450 2d" (Waxman *et al.*, 1985).

studied of the polymorphisms (Mahgoub *et al.*, 1977); other examples include phenacetin *O*-deethylation and mephenytoin 4-hydroxylation. We have previously purified and characterized the human liver P-450s involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation (Distlerath *et al.*, 1985, Distlerath and Guengerich, 1984).

S-Mephenytoin (5-phenyl-5-ethyl-*N*-methylhydantoin) is an anticonvulsive drug which is metabolized by *N*-demethylation and 4-hydroxylation (of the aromatic ring) (Küpfer and Preisig, 1984; Troupin *et al.*, 1976; Küpfer *et al.*, 1981). About 5–10% of Caucasian people display a "slow" metabolism phenotype *in vivo* (Küpfer and Preisig, 1983, 1984; Troupin *et al.*, 1976; Küpfer *et al.*, 1981; Wedlund *et al.*, 1984). In order to better understand the biochemical basis for this polymorphism and develop knowledge that could be applied clinically, we sought to purify this enzyme from human liver and characterize it. In this article, we report the purification of the enzyme from five different liver samples and the biochemical comparison of the different preparations. We also report here a study of the specificity of this enzyme toward a series of hydantoin derivatives as well as other typical P-450 substrates using techniques of enzyme reconstitution and immunochemical inhibition; we compare the steady-state kinetic parameters of two M_r variants of the enzyme; and we explore the role of cytochrome b_5 in the reaction using reconstitution and immunochemical inhibition techniques.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Identity of P-450$_8$ and P-450$_{MP\text{-}1}$ in Human Liver Microsomes—We first purified P-450$_{MP}$ from liver sample HL 31 according to the general method described under "Experimental Procedures," assaying catalytic activity at each step after removal of detergent and reconstitution. In this case, the hydroxylapatite column became severely compressed, and overall recovery of P-450 from the solubilized microsomes was less than 0.5%. However, the purified preparation gave a single protein band on NaDodSO$_4$-polyacrylamide gel electrophoresis and had *S*-mephenytoin 4-hydroxylase activity (because of the presence of residual Emulgen 913 in the preparation, we could not determine the exact turnover number). We examined the immunochemical cross-reactivity of the purified enzyme with other isozymes of human liver P-450 which had already been purified in this laboratory (Wang *et al.*, 1983; Distlerath *et al.*, 1985). Purified P-450$_{MP}$ reacted strongly with antibody raised to P-450$_8$ (Wang *et al.*, 1983) in analysis with immunochemical blotting but did not react with antibodies raised against human P-450$_2$, P-450$_3$, P-450$_4$, P-450$_5$, P-450$_{DB}$, or P-450$_{PA}$ (data not shown). To compare the contents of P-450$_8$ and P-450$_{MP\text{-}1}$ in several human liver microsomes, antibodies raised in rabbits to each antigen were used with immunochemical blotting analysis. The content of P-450$_{MP\text{-}1}$ in 12 different human liver microsomal preparations was found to correlate well with the amounts of P-450$_8$ in these preparations ($r = 0.956$), suggesting that the two P-450s are identical or very similar (data not shown). Fig. 2 shows

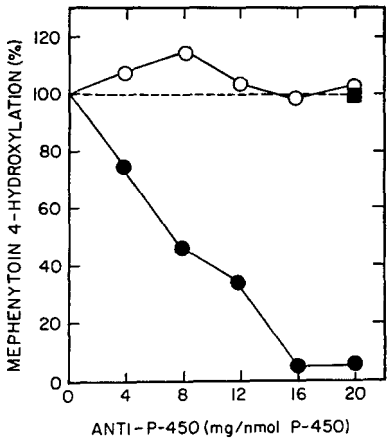

FIG. 2. **Inhibition of mephenytoin 4-hydroxylase activity by anti-P-450$_8$ in human liver microsomes.** Human liver microsomes (sample HL 38) containing 20 pmol of P-450 were preincubated at 23 °C for 10 min with various amounts of anti-P-450$_8$ (●), anti-P-450$_3$ (○), or preimmune antibody (■) IgG fractions. *S*-Mephenytoin 4-hydroxylase activity of the liver microsomes was measured as described under "Experimental Procedures." The activity without IgG was 0.18 nmol of 4-hydroxymephenytoin formed per min/nmol of P-450.

TABLE I

Purification of P-450$_{MP\text{-}1}$ from human liver microsomes

	Protein	P-450		Purification	Yield
		Total	Specific content		
	mg	*nmol*	*nmol/mg protein*	*-fold*	*%*
Microsomes[a]	2400	1830	0.76	1	100
Solubilized supernatant	2230	1800	0.81	1.1	98
Octylamino-Sepharose 4B	135	609	4.5	5.9	33
Hydroxylapatite	19	160	8.6	11	9
DE52	9.8	134	13.7	18	7
CM52	4.8	68	14.3	19	4

[a] Sample HL 36 was used.

that rabbit anti-P-450$_8$ completely inhibited *S*-mephenytoin 4-hydroxylase activity in human liver microsomes. IgG fractions from rabbits immunized with the other human P-450s (*see above*) did not inhibit catalytic activity; in Fig. 2, only the lack of effect of anti-P-450$_3$ is shown for example.

Purification of P-450$_{MP\text{-}1}$ and P-450$_{MP\text{-}2}$—Chromatography was used to purify the P-450 responsible for the 4-hydroxylation of *S*-mephenytoin from human livers, and enzyme fractions were monitored by the use of NaDodSO$_4$-polyacrylamide gel electrophoresis and immunochemical detection of resolved proteins (transferred to nitrocellulose sheets) with rabbit anti-P-450$_{MP}$, as described above (Table I). Hydrophobic affinity chromatography of cholate-solubilized microsomes on a *n*-octylamino-Sepharose 4B column yielded the majority of P-450$_{MP\text{-}1}$ in fractions eluted with the buffer containing 0.06% (w/v) Emulgen 913 (Guengerich and Martin, 1980; Wang *et al.*, 1983; Distlerath *et al.*, 1985). The pooled fractions were purified further by chromatography on a hydroxylapatite column. Most of the P-450$_{MP\text{-}1}$ was routinely found in the hemoprotein peak eluted with 300 mM potassium phosphate buffer (after extensive washing with 40, 90, and 180 mM buffers). Since fractions from this peak contained two major polypeptides and minor polypeptide contaminants as shown in Fig. 3, further purification with Whatman DE52 and CM52 ion-exchange column chromatography was carried out. P-450$_{MP\text{-}1}$ was eluted in the void fraction of DE52 columns under these conditions. This fraction was applied to a CM52 column,

[2] Portions of this paper (including "Experimental Procedures" and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1428, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

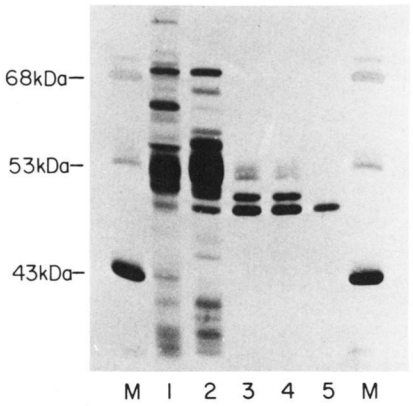

FIG. 3. **NaDodSO$_4$-polyacrylamide gel electrophoresis of purified P-450$_{MP-1}$ fractions of human liver microsomes.** Electrophoresis was carried out with solubilized microsomes (*lane 1*), *n*-octylamino-Sepharose 4B eluate (*lane 2*), hydroxylapatite eluate (*lane 3*), DE52 eluate (*lane 4*), and CM52 eluate (*lane 5*, 2 μg of protein). All samples were derived from sample HL 36 microsomes. The molecular mass standards (*lanes M*) used were bovine serum albumin (accepted 68 kDa), *Escherichia coli* L-glutamate dehydrogenase (53 kDa), and equine liver alcohol dehydrogenase (43 kDa). The anode was at the *bottom* of the figure. The gel was stained with a silver method (Wray *et al.*, 1981).

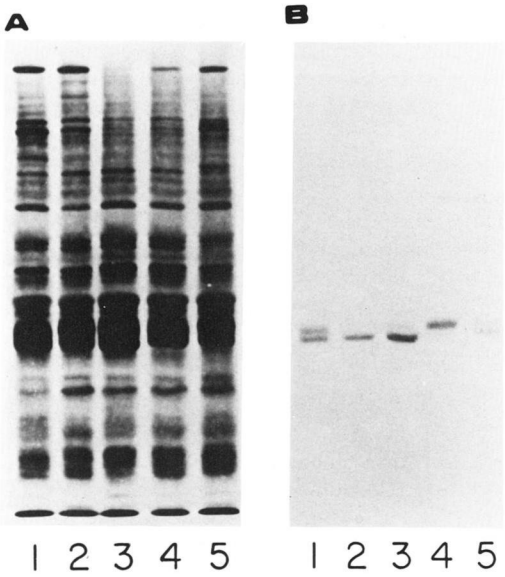

FIG. 4. **NaDodSO$_4$-polyacrylamide gel electrophoresis and immunoblotting of human liver microsomes.** Ten μg of liver microsomes from samples HL 70 (*lane 1*), HL 72 (*lane 2*), HL 31 (*lane 3*), HL 32 (*lane 4*), and HL 33 (*lane 5*) was electrophoresed. The anode was at the *bottom* of the figure. After electrophoresis, the gel was stained with a silver method (Wray *et al.*, 1981) (*A*) or the proteins were transferred to nitrocellulose and treated with anti-P-450$_{MP-1}$ (1:100 dilution of antisera) (*B*).

where P-450$_{MP-1}$ was eluted with a NaCl concentration of about 60 mM. The purified P-450$_{MP-1}$ thus obtained was homogeneous as judged by NaDodSO$_4$-polyacrylamide gel electrophoresis, as shown in Fig. 3, and contained 12–17 nmol of P-450/mg of protein in all cases. The minimum M_r of P-450$_{MP-1}$ was determined to be about 48,000. The fold purification of P-450$_{MP-1}$ was greater than 18 (based on total microsomal P-450), and the apparent recovery from sample 36 liver microsomes was about 4% (Table I).

Fig. 4 shows both NaDodSO$_4$-polyacrylamide gel electrophoresis and immunochemical staining (with anti-P-450$_{MP-1}$) of five human microsomal preparations. With immunoblotting analysis, we detected two polypeptides in some human liver microsomal preparations that reacted with anti-P-450$_{MP-1}$; the low M_r polypeptide is designated P-450$_{MP-1}$ and the higher M_r protein is termed P-450$_{MP-2}$. Of particular interest is the observation that one of the humans (sample HL 32) contained essentially no P-450$_{MP-1}$ but only P-450$_{MP-2}$. We purified this protein using the same chromatographic procedures described above. Using immunoblotting, we found that both proteins have the same chromatographic properties on hydroxylapatite and DEAE- and CM-cellulose and react with anti-P-450$_{MP-1}$ with almost equal intensity. As shown in Fig. 5, P-450$_{MP-2}$ from sample HL 32 was homogeneous on NaDodSO$_4$-polyacrylamide gel electrophoresis and had a minimum M_r of about 50,000. When the P-450$_{MP-2}$ was mixed with P-450$_{MP-1}$, the two P-450s could be clearly separated by NaDodSO$_4$-polyacrylamide gel electrophoresis.

Spectral Properties of Cytochromes P-450$_{MP-1}$ and P-450$_{MP-2}$—Fig. 6 shows the spectral properties of P-450$_{MP-1}$ (from sample HL 38) and P-450$_{MP-2}$ (from sample HL 32). Both P-450s were found to have similar spectral properties, *i.e.* in the oxidized states, both proteins had an α band at 567–568 nm, a β band at 534–536 nm, and a Soret peak at 416 nm, and the wavelength maximum of the reduced carbon monoxide complex was found to be 449.5 nm in both cases. The ferric proteins appear to be mainly low-spin. The two preparations (and others) contained relatively little cytochrome P-420.

Amino Acid Compositions—The amino acid compositions of two preparations of P-450$_{MP-1}$ (from samples HL 36 and HL 38) and one preparation of P-450$_{MP-2}$ (from sample HL 32) are presented in Table II. These compositions are based only on 24-h hydrolysis and do not include cysteine and tryptophan values; the compositions are calculated on the basis of apparent M_r values of 48,000 and 50,000 for P-450$_{MP-1}$ and P-450$_{MP-2}$, respectively.

The amino acid compositions of these preparations were found to be very similar except that the methionine content in the P-450$_{MP-2}$ preparation was somewhat less than that in P-450$_{MP-1}$ preparations.

The difference indices (Metzger *et al.*, 1968) for the three preparations were calculated and compared with other human P-450s, including P-450$_{DB}$ and P-450$_{PA}$. The difference index for two P-450$_{MP-1}$ preparations (samples HL 36 and HL 38) was only 2.7, indicating similar amino acid composition. The indices between P-450$_{MP-1}$ and P-450$_{MP-2}$ were calculated to be 2.7 and 3.7, suggesting that the two proteins are also very

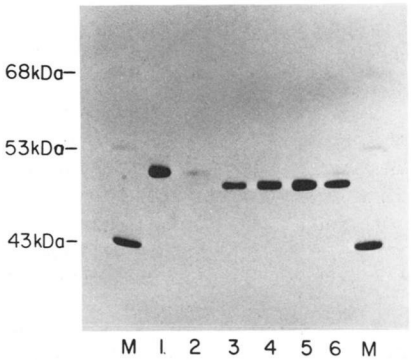

FIG. 5. **NaDodSO$_4$-polyacrylamide gel electrophoresis of purified human P-450$_{MP}$ preparations used.** P-450$_{MP-1}$ from samples HL 36 (*lane 3*), HL 37 (*lane 4*), HL 38 (*lane 5*), and HL 91 (*lane 6*) and P-450$_{MP-2}$ from samples HL 32 (*lane 1*) and HL 33 (*lane 2*) are shown. Molecular mass standards (*lanes M*) were also used. Conditions for electrophoresis were as described in the legend to Fig. 3.

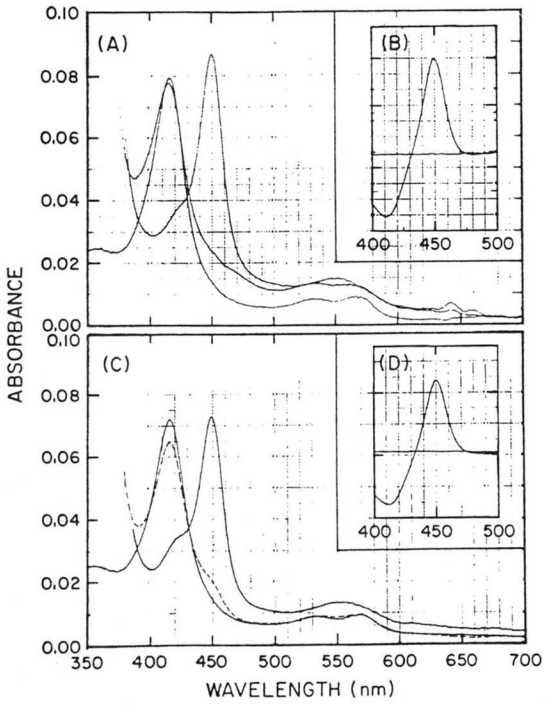

FIG. 6. **Spectral properties of P-450$_{MP-1}$ and P-450$_{MP-2}$.** Spectra of P-450 (in 100 mM potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) sodium cholate, and 0.4% (w/v) Emulgen 913) were obtained with a Cary 219 spectrophotometer in the automatic base-line correction mode. The absolute spectra of P-450$_{MP-1}$ (from sample HL 38) and P-450$_{MP-2}$ (from sample HL 32) are shown in *A* and *B*, respectively (Fe^{3+}, Fe^{2+}, and Fe^{2+}-CO). The *insets* (*B* and *D*) show Fe^{2+}-CO *versus* Fe^{2+} difference spectra of each purified P-450. Reduction was done by the addition of solid $Na_2S_2O_4$.

TABLE II

Amino acid compositions of P-450$_{MP-1}$ and P-450$_{MP-2}$

Amino acid	Number of residues[a]		
	P-450$_{MP-1}$		P-450$_{MP-2}$, sample HL 32
	Sample HL 36	Sample HL 38	
Ala	27	26	28
Arg	16	19	17
Asx	40	40	43
Glx	55	54	58
Gly	53	57	61
His	12	14	13
Ile	21	19	21
Leu	36	33	37
Lys	24	22	25
Met	7	7	4
Phe	19	18	19
Pro	19	22	21
Ser	58	56	55
Thr	21	21	24
Tyr	11	12	11
Val	26	27	26
(Total)[b]	(445)	(447)	(463)

[a] Values presented are means of duplicate determinations.
[b] Not including cysteine or tryptophan.

similar. P-450$_{MP-1}$ and P-450$_{MP-2}$ had different amino acid compositions than P-450$_{DB}$ and P-450$_{PA}$, for the indices between them were calculated to be greater than 6. When we compared P-450$_{MP-1}$ and P-450$_{MP-2}$ to the reported compositions of rat P-450s (Haniu *et al.*, 1984), the difference indices were all in the range of 5.5–12.

Peptide Mapping of P-450$_{MP-1}$ and P-450$_{MP-2}$—Partial digestion by proteases (*Staphylococcus aureus* V8 protease, α-chymotrypsin, or papain) followed by NaDodSO$_4$-polyacrylamide gel electrophoresis was also used to compare P-450$_{MP-1}$ and P-450$_{MP-2}$. Fig. 7 shows that limited proteolysis of these two forms of P-450 gave very similar peptide maps, but some slightly different cleavage products were obtained. Particularly in the case of digestion by papain, some differences in polypeptides were noted. The same conclusion was reached from a comparison of the tryptic peptides using high performance liquid chromatography. The tryptic peptides obtained from P-450$_{MP-1}$ (samples HL 36 and HL 38) and P-450$_{MP-2}$ (sample HL 32) were very similar (data not shown).

Double Immunodiffusion Analysis—Rabbit antisera against P-450$_{MP-1}$ and P-450$_{MP-2}$ were prepared separately. Double immunodiffusion analysis was performed to examine the immunochemical relationship of P-450$_{MP-1}$ and P-450$_{MP-2}$. As shown in Fig. 8, anti-P-450$_{MP-1}$ (P-450$_{MP-1}$ from sample HL 38) formed a single precipitin line with both P-450$_{MP-1}$ from samples HL 36, HL 37, HL 38, and HL 91, and anti-P-450$_{MP-2}$ (P-450$_{MP-2}$ from sample HL 32) gave the almost same results obtained with anti-P-450$_{MP-1}$. The same results were obtained with either antiserum and with detergent-solubilized human liver microsomes. Both antisera formed single precipitin lines (and no spurs) with the solubilized microsomes from samples HL 38 and HL 32 as well as with purified P-450$_{MP-1}$ (sample HL 38) and P-450$_{MP-2}$ (sample HL 32), consonant with high immunochemical similarity. No spurs were observed when the anti-P-450$_8$ preparation (Wang *et al.*, 1983; *see below*) was compared with anti-P-450$_{MP-1}$ or anti-P-450$_{MP-2}$.

In Vitro Translation of P-450$_{MP-1}$ and P-450$_{MP-2}$—None of the preceding studies really distinguished P-450$_{MP-1}$ from P-450$_{MP-2}$, with the exception of NaDodSO$_4$-polyacrylamide gel electrophoresis. The possibility existed that P-450$_{MP-1}$ and P-450$_{MP-2}$ differ only in post-translational modifications or are artifacts arising from post-mortem changes or storage. We isolated RNA from four human liver samples and translated proteins in the presence of [^{35}S]methionine and a rabbit reticulocyte lysate system. Anti-P-450$_{MP-1}$ was used to precipitate products of interest using a solid-phase method; the recovered material was electrophoresed, and a fluorograph is

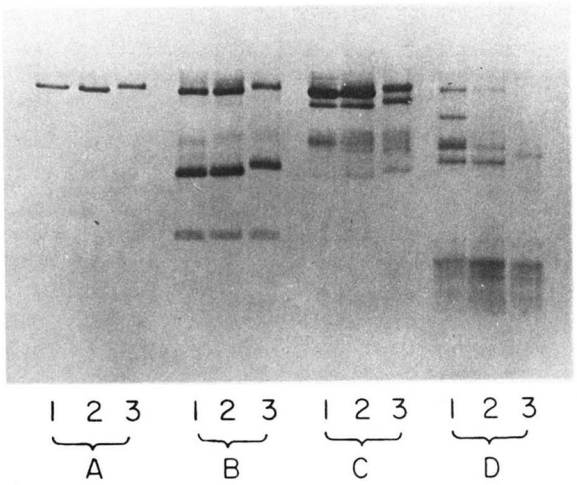

FIG. 7. **Comparison of proteolytic digests of purified P-450$_{MP-1}$ and P-450$_{MP-2}$ using NaDodSO$_4$-polyacrylamide gel electrophoresis.** The P-450s were undigested in *A* or were digested with *S. aureus* V8 protease (*B*), α-chymotrypsin (*C*), or papain (*D*) as described under "Experimental Procedures." *Lane 1* contained P-450$_{MP-1}$ (from sample HL 38), *lane 2* contained P-450$_{MP-1}$ (from sample HL 36), and *lane 3* contained P-450$_{MP-2}$ (from sample HL 32). The gel was stained with silver (Wray *et al.*, 1981).

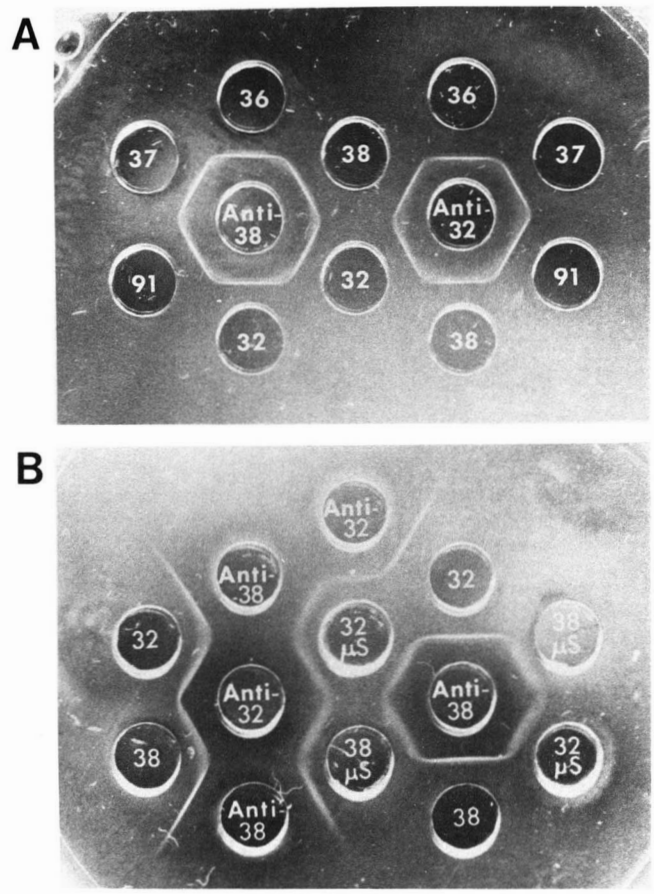

FIG. 8. **Comparison of P-450$_{MP-1}$ and P-450$_{MP-2}$ by double immunodiffusion analysis.** *Anti-38* and *anti-32* represent the rabbit antisera raised to P-450$_{MP-1}$ (from sample HL 38) and P-450$_{MP-2}$ (from sample HL 32), respectively. In *A*, four preparations of purified P-450$_{MP-1}$ (from samples HL 36, HL 37, HL 38, and HL 91) and one preparation of purified P-450$_{MP-2}$ (from sample HL 32) were included. In *B*, the solubilized microsomes from samples HL 38 and HL 32 were applied as well as the purified preparations of P-450$_{MP-1}$ (sample HL 38) and P-450$_{MP-2}$ (sample HL 32).

presented in Fig. 9. RNA isolated from an individual containing primarily only P-450$_{MP-2}$ (sample HL 32) produced only a labeled polypeptide with the same apparent monomeric M_r as P-450$_{MP-2}$ (50,000), and RNA isolated from an individual containing only P-450$_{MP-1}$ (sample HL 93) produced only a labeled polypeptide with the sample apparent M_r as P-450$_{MP-1}$ (48,000). (Both samples HL 32 and HL 93 also yielded traces of a lower M_r ^{35}S-product which is not thought to be related to P-450$_{MP}$.) Liver samples HL 91 and HL 92 each contained both P-450$_{MP-1}$ and P-450$_{MP-2}$ (Fig. 9); the RNA samples prepared from these two particular samples yielded only low levels of total translation products (Fig. 9*A* shows very weak bands in the position of P-450$_{MP-1}$). The apparent translation frequency was estimated to be in the range of 0.05% in all four cases.

N-terminal Amino Acid Sequences of P-450$_{MP-1}$ and P-450$_{MP-2}$—The sequences determined using automated Edman degradation are shown in Fig. 10, and yield data are given in Table III. The sequences were identical at all residues which could be defined with certainty. The yields of the PTH derivatives of tyrosine and tryptophan at positions 10 and 13 were rather low in P-450$_{MP-1}$, and positions 10 and 13 in P-450$_{MP-2}$ were not identified. Cysteine products could not be found, even after prior performate oxidation or reduction and carboxymethylation with iodo[^{14}C]acetic acid. Fig. 10 also shows comparisons with reported sequences of rat and rabbit P-450s (Haniu *et al.*, 1984; Black and Coon, 1985). The most homologous proteins were rat P-450$_{UT-A}$, P-450$_{UT-I}$, P-450$_{PB-C}$, and P-450$_f$. These rat proteins have 10–15 of the first 20 residues in common with P-450$_{MP}$. Rat P-450$_g$ showed 9 of the first 15 residues. The other rat P-450s seem to be less related, and little homology to the rabbit proteins was noted, except for P-450$_{LM-2}$ and P-450$_{LM-3b}$ (7 and 8 of the first 20 residues, respectively, were identical).

Reconstitution of Mephenytoin 4-Hydroxylase Activity by Purified P-450$_{MP-1}$ and P-450$_{MP-2}$ and the Effects of Rat and Human Cytochrome b_5—*S*-Mephenytoin 4-hydroxylase activity was reconstituted in a system containing purified P-450$_{MP-1}$ or P-450$_{MP-2}$, NADPH-cytochrome-P-450 reductase, and L-α-dilauroyl-*sn*-glyceryo-3-phosphocholine. The activity was dependent on the incubation time (Fig. 11*A*) and on the presence of NADPH-cytochrome-P-450 reductase (Fig. 11*B*) and purified P-450$_{MP-1}$ (in this case, the preparation was used from liver microsomal sample HL 37) (Fig. 11*D*). L-α-Dilauroyl-*sn*-glyceryo-3-phosphocholine activated the hydroxylase activity about 2-fold with the optimal effect at a concentration of 40–50 μg/ml (Fig. 11*C*).

Addition of rat cytochrome b_5 to the complete reconstituted system caused further stimulation of *S*-mephenytoin 4-hydroxylase activity; a 3-fold increase in the activity was obtained with a molar ratio of cytochrome b_5 to P-450$_{MP-1}$ of 2–3 (Fig. 12*A*). Similar results were obtained when the rat cytochrome b_5 was replaced by human cytochrome b_5 which had been treated extensively to remove any residual detergent (Fig. 12*B*).

Table IV compares the *S*-mephenytoin 4-hydroxylase activities of the reconstituted monooxygenase system containing four different preparations of P-450$_{MP-1}$ and one preparation of P-450$_{MP-2}$ with the activities of the respective human liver microsomes. The effects of cytochrome b_5 on the activities of the five P-450 preparations are also included in Table IV in some instances. In the presence of cytochrome b_5, the activities of the reconstituted systems were higher than the respective human liver microsomes. (Liver microsomes from sample HL 91 contained higher activities than the reconstituted system devoid of cytochrome b_5.) A maximal purification factor of 6 (based on turnover numbers derived from total P-450 concentrations) was observed when the reconstituted system containing P-450$_{MP-1}$ from sample HL 36 liver microsomes was used. P-450$_{MP-2}$ seemed to have only slightly lower mephenytoin hydroxylase activity than P-450$_{MP-1}$. Cytochrome b_5 stimulated the *S*-mephenytoin 4-hydroxylase activity in all of the reconstituted systems containing P-450$_{MP-1}$ or P-450$_{MP-2}$.

Fig. 13 shows the kinetic analysis of mephenytoin 4-hydroxylase activity of reconstituted systems containing five different preparations of purified P-450$_{MP}$ in the presence of cytochrome b_5. The K_m value was estimated to be about 1.25 mM in all of the systems containing the different preparations of P-450$_{MP}$. The V_{max} value varied from 0.83 to 1.25 nmol of 4-hydroxymephenytoin formed per min/nmol of P-450.

Inhibition of Mephenytoin 4-Hydroxylase Activity of Human Liver Microsomes by Anti-human Cytochrome b_5—In order to better address the possible role of cytochrome b_5 in 4-hydroxylation of mephenytoin, the effect of anti-human cytochrome b_5 on the activity was examined. The antibody raised to purified human liver cytochrome b_5 recognized only a single polypeptide in three different human liver microsomal preparations as judged by immunoblotting analysis (*e.g.* Fig. 1). Since in the NADH-dependent electron transfer system cy-

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