A Phenobarbital-Inducible Hepatic Mitochondrial Cytochrome P-450 Immunochemically Related to Microsomal P-450b†

Rass M. Shayiq and Narayan G. Avadhani*

Laboratories of Biochemistry, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, Pennsylvania 19104-6048

Received July 14, 1989; Revised Manuscript Received September 18, 1989

ABSTRACT: We have purified and characterized a phenobarbital (PB)-inducible hepatic mitochondrial cytochrome P-450 (P-450), termed P-450mt4, which is distinctly different from the previously characterized mitochondrial isoforms. The level of induction of P-450mt4 by PB in the male livers is nearly 20-fold, as against a marginal induction in the female livers, suggesting that it may be a male predominant isoform. P-450mt4 shows a close resemblance to microsomal P-450b (the major PB-inducible form) with respect to electrophoretic migration (apparent molecular mass of 50 kDa) and immunological cross-reactivity, although it exhibits a distinct isoelectric pH (p*I* 6.9 vs 6.5 for P-450b), peptide fingerprint pattern, and amino acid composition. Further, the N-terminal sequence analysis shows over 90% positional identity (39 out of 42) between P-450mt4 and P-450b, suggesting that it is a close relative of the P-450 IIB gene family. In vitro reconstitution experiments show that P-450mt4 can metabolize a wide range of substrates such as benzphetamine, (dimethylamino)antipyrine, aflatoxin B_1, and vitamin D_3, exclusively in the presence of mitochondrial-specific ferredoxin and ferredoxin reductase as electron carriers. P-450mt4 is translated as a 53-kDa precursor, which is transported into mitochondria under in vitro conditions and processed into a mature 50-kDa protein. These results provide conclusive evidence for the occurrence of a male-specific P-450 belonging to the IIB gene family in rat liver mitochondria.

A multitude of structurally diverse physiological substrates, as well as xenobiotic compounds, are metabolized in various animal tissues by a cytochrome P-450 (P-450)[1]-type mixed-function oxygenase system. The diversity in substrate specificity has been attributed to the presence of multiple species of P-450 in the endoplasmic reticulum of liver and other tissues (Ryan et al., 1982; Gelboin, 1980; Conney, 1982; Whitlock, 1986; Guengerich et al., 1982, 1987). On the basis of the pattern of induction by known P-450 inducers like clofibrate, steroids, phenobarbital, polycyclic aromatic hydrocarbons, etc., and structural similarities to the major isoforms induced by these compounds, the P-450 super gene family has been classified into a number of major families (Nebert et al., 1987; Nebert & Gonzalez, 1987). The PB-related subfamily, designated as IIB, seems to include 10–20 closely related genes (Atchison & Adesnik, 1983; Adesnik & Atchison, 1986; Nebert & Gonzalez, 1987; Rampersaud & Walz, 1987; Giachelli et al., 1989) which share 50–97% homology at the level of DNA and protein sequences (Fugii Kuriyama et al., 1982; Yuan et al., 1983; Sogawa et al., 1984). All members of the IIB subfamily isoenzymes thus far identified, including a non-heme protein of unknown function (Kimura et al., 1989), have been localized to the hepatic microsomal fraction.

It is now well established that mitochondria from various steroidogenic tissues (Takikawa et al., 1978; Simpson, 1979; Jefcoate, 1977; Kashiwagi et al., 1980), kidney (Gray et al., 1972; Ghazarian et al., 1974; Hitwatashi et al., 1982), and liver (Bjorkhem & Gustafsson, 1974; Sato et al., 1977; Pedersen et al., 1979; Niranjan & Avadhani, 1980; Wikvall, 1984; Niranjan et al., 1984) contain cytochrome P-450. Recent experiments reported from our laboratory also showed that BNF (a 3-methylcholanthrene type of inducer) and PB induce at least three different isoenzymic forms of P-450 in rat liver mitochondria which exhibit varying levels of vitamin D_3 25-hydroxylase and C-27 sterol 26-hydroxylase activities, and distinctive abilities to metabolize structurally diverse carcinogens and xenobiotic agents (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989). It was also shown that one of the BNF-inducible isoforms termed P-450mt2 exhibits immunochemical similarity with the similarly induced microsomal P-450c (the major BNF-inducible isoform). These two isoforms, however, showed distinctive differences with respect to posttranslational processing of the apoprotein molecules (Niranjan et al., 1988), peptide fingerprints, substrate specificity, electron-transfer protein requirement for the reconstitution of enzyme activity (Raza & Avadhani, 1988), and N-terminal amino acid sequences (R. M. Shayiq and N. G. Avadhani, unpublished results). Hepatic mitochondrial isoforms P-450mt1 (BNF induced) and P450mt3 (PB induced), on the other hand, share no detectable immunochemical homology with similarly induced microsomal P-450 isoforms (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989). In the present study, we have purified another hepatic mitochondrial isoform termed P-450mt4 which is profusely induced by PB in the male and weakly in the female rat livers. Our results demonstrate that despite showing immunochemical relatedness to the microsomal P-450b (the major PB-inducible form), P-450mt4 represents an independent mitochondrial species.

Experimental Procedures

Materials. *d*-Benzphetamine hydrochloride, aflatoxins B_1, M_1, Q_1, and P_1, ω-octylamine–agarose, (dimethylamino)-antipyrine, sodium cholate, poly(ethylene glycol) (molecular mass 8 kDa), NADP, isocitrate, isocitrate dehydrogenase,

† This research was supported in part by U.S. Public Health Service Grants CA-22762 and GM-34883.

* To whom correspondence should be addressed.

[1] Abbreviations: P-450, cytochrome P-450; BNF, β-naphthoflavone; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); Fdx, dog hepatic mitochondrial ferredoxin; Fdr, dog hepatic mitochondrial ferredoxin reductase; HPLC, high-pressure liquid chromatography.

0006-2960/90/0429-866$02.50/0

vitamin D_3, dilaurylphosphatidylcholine, V_8 protease, and Lubrol Px were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxylapatite (Biogel HTP), Biobeads SM-2 (20–50 mesh), and chemicals for polyacrylamide gel electrophoresis and isoelectric focusing were obtained from Bio-Rad Laboratories (Richmond, CA). Phenobarbital (Na salt) was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Nitrocellulose membrane for protein transfer was purchased from Schleicher & Schuell Corp. (Keene, NH), and all other reagents for the Western blot analysis were from Promega Biotech Corp. (Madison, WI). IgG sorb (protein A coated cells) was from The Enzyme center (Malden, MA). [^{3}H]-Vitamin D_3 (35–60 Ci/mmol) and [^{3}H]-25-hydroxy-vitamin D_3 (150–170 Ci/mmol) were from New England Nuclear Corp. (Boston, MA). [^{35}S]Methionine (800 Ci/mmol) was from Amersham Radiochemical Corp. (Arlingten Heights, IL), and [^{3}H]AFB_1 (16 Ci/mmol) was from Moravek Biochemicals (Breer, CA). All other reagents used were of highest purity grades obtained from Fluka Chemical Corp. (Ronkonkoma, NY), Sigma Chemical Co. (St. Louis, MO), or Fisher Scientific Co. (Pittsburgh, PA).

Isolation of Subcellular Fractions and Enzyme Purification. Mitochondria and microsomes were isolated from livers of male Sprague Dawley rats (125–150 g) treated with PB for 5 days (75 mg kg^{-1} day^{-1}) as described previously from this laboratory (Niranjan et al., 1984; Raza & Avadhani, 1988). Mitochondria were treated with digitonin and washed 2 times with mitochondrial isolation buffer to obtain mitoplasts containing less than 1% microsomal contamination as described before (Bhat et al., 1982; Niranjan et al., 1984). P-450 from PB-induced hepatic mitoplasts was solubilized by sonic disruption followed by cholate extraction and enriched by precipitation with 15% PEG (Raza & Avadhani, 1988). P-450mt4 was purified by using chromatography on ω-octylamine–agarose, DEAE-Sephacel, and hydroxylapatite essentially as described for the purification of P-450mt3 (Shayiq & Avadhani, 1989) except that P-450 from the ω-octylamine–agarose column was eluted with a buffer containing 0.06% Lubrol-Px instead of 0.2% used in our previous purifications. Purification of P-450b from PB-induced hepatic microsomes and isolation of electrophoretically homogeneous Fdx and Fdr from dog liver mitochondria were carried out as described recently (Shayiq & Avadhani, 1989). NADPH–cytochrome P-450 reductase was purified from rat liver microsomes by affinity binding to 2′,5′-ADP–agarose as described by Yasukochi and Masters (1976). Polyclonal antibodies to P-450b and P-450mt3 were raised in rabbits and made monospecific by affinity adsorption to purified antigen immobilized on nitrocellulose membrane.

In Vitro Reconstitution. The purified P-450mt4 and P450b were reconstituted in 0.5-mL volumes with NADPH–cytochrome P-450 reductase, or with Fdx and Fdr electron-transfer systems as described before (Niranjan et al., 1984; Shayiq & Avadhani, 1989). The reaction was initiated by adding 500 nmol of *d*-benzphetamine, or 2.5 μmol of (dimethylamino)-antipyrine, or 100 nmol of [^{3}H]aflatoxin B_1 (2200 dpm/nmol), or 100 nmol of [^{3}H]vitamin D_3 (11000 dpm/nmol) and carried out for 10–30 min as needed. (Dimethylamino)antipyrine demethylase and *d*-benzphetamine demethylase activities were measured spectrophotometrically as described by Cochin and Axelrod (1959) and Nash (1953). AFB_1 metabolites were extracted into $CHCl_3$ and analyzed by HPLC as described recently (Shayiq & Avadhani, 1989). Vitamin D_3 25-hydroxylase activity was assayed by normal-phase and reverse-phase HPLC as described before (Burgos-Trinidad et al., 1986; Raza & Avadhani, 1988).

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Protein samples were subjected to electrophoresis on 12% SDS–polyacrylamide gels and visualized by staining with Coomassie Blue. For Western blot analysis, proteins from unstained gels were electrophoretically transferred to a nitrocellulose membrane and probed with appropriate primary antibody (Towbin et al., 1979). The immunoreactive bands were identified by using alkaline phosphatase conjugated secondary antibody and chromogenic substrates supplied as a kit by Promega Biotech Corp.

Two-dimensional polyacrylamide gel electrophoresis was carried out essentially as described by O'Farrell (1975) using the modifications described in a recent report (Niranjan et al., 1988). The immunoreactive proteins on the gel were detected by using the Western blot method described above.

Peptide Mapping. Purified P-450mt4 and P-450b (6–10 μg of protein for each digestion) were subjected to limited proteolysis with 5 μg of V_8 protease for 5 and 10 min using incubation conditions of Cleveland et al. (1977). The reaction was stopped by boiling in the presence of 4% 2-mercaptoethanol and 5% SDS, and the peptide fragments were resolved on 16% SDS–polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane and probed with antibody to P-450b by the Western blot procedure.

N-Terminal Sequence Analysis. P-450mt4 protein (40–60 pmol) was electrophoretically transferred onto a PVDF Immobilon membrane (Millipore Corp.) and sequenced in an Applied Biosystems Model 475A gas-phase sequencer by the phenylthiohydantoin procedure. The conditions of gel electrophoresis and transfer were as described by Matsudaira (1987) and Moos et al. (1988). The sequencing was carried out in Dr. Audree Fowler's facility at UCLA, School of Medicine, Los Angeles, CA.

In Vitro Transport of Proteins into Mitochondria. Poly(A) RNAs from membrane-free polyribosomes of PB-induced rat liver were isolated as described by Bhat et al. (1982). In vitro translation of RNA in rabbit reticulocyte lysates using 1.0 μCi/μL [^{35}S]methionine, in vitro transport of translation products into rat liver mitochondria, and immunoprecipitation of labeled proteins was performed as described in a recent paper (Niranjan et al., 1988).

Results

Purification of P-450. The mitochondrial P-450 was solubilized by sonication and cholate extraction, and purified by using the combination of hydrophobic and ion-exchange column chromatography as described recently for the purification of two BNF-inducible, one PB-inducible, and a control isoform from noninduced hepatic mitochondria (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989). As observed during the purification of P-450mt3, the heme proteins from PB-induced rat liver mitochondria elute from the ω-octylamine–agarose column (in the presence of 0.2% Lubrol) as a broad complex peak (Shayiq & Avadhani, 1989). The relatively more hydrophobic second half of the peak was used for the purification of P-450mt3 (Shayiq & Avadhani, 1989). Although not shown here, the less hydrophobic first half of the peak can be selectively eluted with buffer containing 0.06% Lubrol, leaving behind the more hydrophobic P-450mt3. Figure 1A shows the electrophoretic patterns of protein at various stages of P-450 purification. The PEG fraction (lane 2) contains a number of proteins including two prominent species of 52- and 50-kDa molecular mass. In keeping with the differential elution of heme protein from the ω-octylamine–agarose column described above, the protein fraction

Table I: Purification of P-450mt4 from PB-Induced Rat Hepatic Mitochondria

fraction	total protein (mg)	cyt P-450 (nmol)	sp content (nmol/mg of protein)	*x*-fold purification	yield (%)
mitoplast	3520	731	0.207		
15% PEG	1700	535	0.315	1.52	73.0
ω-octylamine–agarose (0.06% Lubrol eluant)	167	178	1.07	5.1	24.3
DEAE-Sephacel	17	96	5.65	27.2	13.1
hydroxylapatite	3.2	48	15.0	72.4	6.5

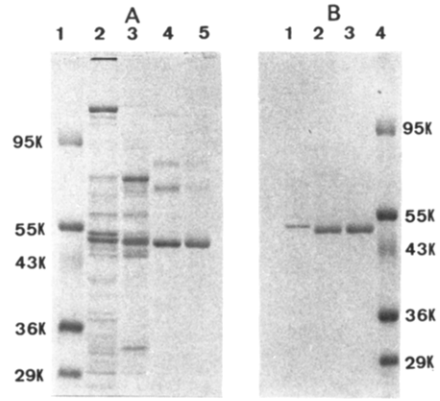

FIGURE 1: Electrophoretic patterns of P-450mt4 at different stages of purification. P-450 was purified from PB-induced hepatic mitochondria from male rats. Proteins were dissociated at 95 °C in Laemmli's sample buffer containing 5% 2-mercaptoethanol and 4% SDS and subjected to electrophoresis on 12% SDS–polyacrylamide gels as described under Experimental Procedures. (A) Lane 1, molecular weight markers; lane 2, 50 μg of mitochondrial 15% PEG fraction; lane 3, 20 μg of octylamine–agarose fraction eluted with 0.06% Lubrol containing buffer; lane 4, 10 μg of protein from DEAE fraction; lane 5, 2 μg of protein from hydroxylapatite fraction. (B) Lane 1, 0.7 μg of P-450mt3; lane 2, 2 μg of P-450mt4; lane 3, 2 μg of P-450b; lane 4, molecular weight markers. The bands were visualized by staining with Coomassie Blue.

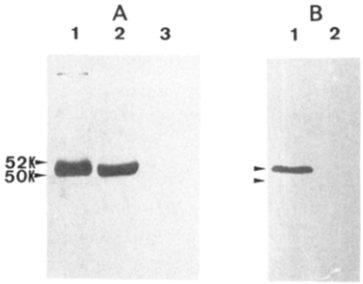

FIGURE 2: Western blot analysis of P-450mt4. About 1–2 μg of purified P-450 isoenzymes was resolved by electrophoresis on a 12% SDS–polyacrylamide gel as described in Figure 1; the proteins were electrophoretically transfered to a nitrocellulose membrane and probed with the appropriate antibodies. (A) Lane 1, P-450b; lane 2, P-450mt4; lane 3, P-450mt3; lane 4, control mt P-450. (B) Lane 1, P-450mt3; lane 2, P-450mt4. The lanes in (A) were probed with anti-P-450b IgG (1:2000 dilution), and the lanes in (B) were probed with anti-P-450mt3 IgG (1:1000 dilution). Other details were as described under Experimental Procedures.

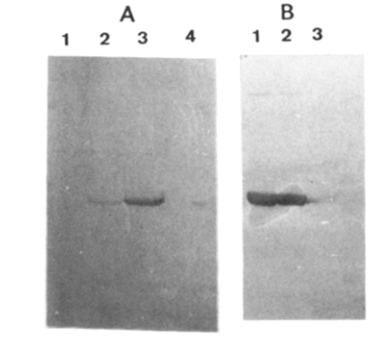

FIGURE 3: Sex-specific induction of hepatic mitochondrial P-450mt4 by phenobarbital. Mitochondria were isolated from control and PB-treated male and female livers, purified by banding in discontinuous sucrose gradients as described by Bogenhagen and Clayton (1974), and treated with digitonin to reduce possible microsomal contamination. Microsomes from treated and untreated male and female livers were prepared by differential centrifugation as described under Experimental Procedures. The PEG fractions from resulting mitoplasts (80 μg of protein in each case) and microsomes (40 μg of protein in each case) were subjected to Western blot analysis using anti-P-450b IgG as the probe. In (A), mitochondrial PEG fractions from uninduced female liver (lane 1), PB-induced female liver (lane 2), PB-induced male liver (lane 3), and uninduced male liver (lane 4) were analyzed. In (B), microsomal proteins from PB-induced female livers (lane 1), PB-induced male liver (lane 2), and uninduced male liver (lane 3) were analyzed.

eluted with 0.06% Lubrol buffer contains predominantly the 50-kDa species and negligible 52-kDa species (see lane 3). Further chromatography of the octylamine–agarose fraction on DEAE and hydroxylapatite columns selectively enriches the 50-kDa heme protein as shown in lanes 4 and 5, respectively. Although not shown, the purified protein showing ≤90% homogeneity (Figure 1, lane 5) exhibits a characteristic CO reduced spectrum with an absorption maximum at 448 nm, and as shown in Table I, this preparation shows a P-450 content of 15 nmol/mg of protein. The P-450 contents at various stages of purification listed in Table I indicate a 72-fold purification with a final yield of 6.5%. For the sake of presentation, the present P-450 from PB-induced mitochondria is referred to as P-450mt4.

Figure 1B shows electrophoretic comparison of P-450mt4 (lane 2) with similarly induced mitochondrial P-450mt3 (lane 1) and microsomal P-450b (lane 3). It is seen that P-450mt4 and P-450b migrate at identical rates with an apparent molecular mass of 50 kDa, while P-450mt3 described in our recent study (Shayiq & Avadhani, 1989) exhibits an apparent molecular mass of 52 kDa.

Immunochemical Properties of P-450mt4. The purified P-450mt4 was compared with similarly induced microsomal P450b, mitochondrial P-450mt3, and uninduced control P-450 by the Western blot analysis. As shown in Figure 2A, antibody to P450b cross-reacted with both P-450b (lane 1) and P450mt4 (lane 2) at comparable intensity, although it failed to cross-react with P-450mt3 and control mt P-450 (lanes 3 and 4). Similarly, as shown in Figure 2B, antibody to P-450mt3 cross-reacts well with the homologous antigen (lane 1) but fails to react with P-450mt4. Further, as reported before, antibody to P-450mt3 cross-reacts with control mt P-450, but not with P-450b (Shayiq & Avadhani, 1989). These results demonstrate that P-450mt4 is immunochemically related to microsomal P-450b, though it is distinctly different from similarly induced mitochondrial P-450mt3. The immunoblot presented in Figure 3A shows the occurrence of an anti-P-450b IgG reactive 50-kDa protein in gradient-purified mitoplasts from PB-induced female and male liver (lanes 2 and 3) and very low to negligible amounts in mitochondria from uninduced female and male livers (lanes 1 and 4). A quantitative analysis of these immunoblots by scanning through an LKB scanner indicates that the extent of induction in the female liver mitochondria (lane 2) is only marginal (3–4-fold) while that in the male liver (lane 3) is about 15–20-fold of control (lane 4),

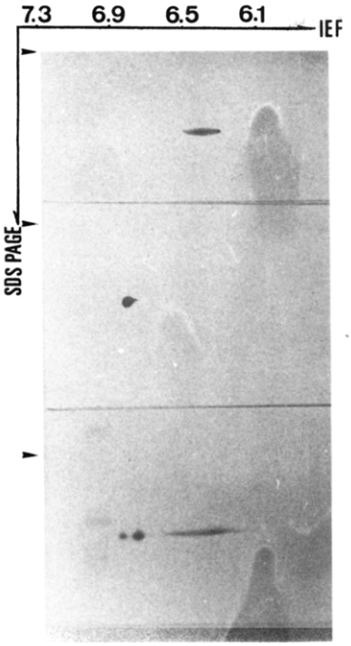

FIGURE 4: Two-dimensional analysis of P-450mt4 and P-450b. One microgram each of P-450b (top), P-450mt4 (middle), and a mixture of the two isoforms (bottom) was subjected to O'Farrell's (1975) two-dimensional analysis. The proteins resolved on the SDS–polyacrylamide gels in the second dimension were transblotted to a nitrocellulose membrane and developed with anti-P-450b IgG and alkaline phosphatase conjugated second antibody as described under Experimental Procedures. The top of the SDS–polyacrylamide gel in the second dimension in each case has been indicated with an arrow.

suggesting sex specificity of P-450mt4 induction. Although not presented, essentially identical Western blot patterns are obtained with mitoplasts prepared from the conventional differential centrifugation method. These results together suggest that the antibody-reactive P-450mt4 is induced preferentially in the male livers by PB.

Molecular Characteristics of P-450mt4. Because of similarity in size and immunochemical cross-reactivity, P-450mt4 was extensively compared with similarly induced microsomal P-450b using multiple approaches. Figure 4 shows the comparison of the two P-450 isoforms by O'Farell-type two-dimensional gel electrophoresis. It is seen that P-450b resolves in the first dimension with an apparent isoelectric pH of 6.5 (Figure 4, top) while P-450mt4 resolves with an apparent isoelectric pH of about 6.9 (Figure 4, middle), though both isoforms show a similar electrophoretic migration in the second dimension (SDS gel). A mixture of P-450b and P-450mt4 (Figure 4, bottom) resolves into distinct components, further confirming that the two proteins differ with respect to isoelectric pH. Differences between these two P-450 isoforms were further verified by peptide fingerprint analysis. In the experiment presented in Figure 5, undigested proteins (lanes 1 and 2) or P-450 digested with V_8 protease for intervals of 5 and 10 min was resolved by electrophoresis, and anti-P-450b IgG-reactive peptides were identified by Western blot analysis. It is seen that the antibody-reactive peptide patterns of P-450b (lanes 3 and 5) and P-450mt4 (lanes 4 and 6) exhibit distinct differences in that the P-450mt4 yields considerably shorter fragments than P-450b. Furthermore, even among the shorter fragments, there are discrete variations between the two isoforms as indicated by the arrows.

The differences with respect to isoelectric pH and fingerprint pattern are further supported by the amino acid composition of P-450mt4 and microsomal P-450b. Results presented in Table II show that the two isoforms exhibit significant variations with respect to Gly, Leu, and Met contents and a close similarity in their Ala, Thr, Asn/Asp, Glu/Gln, Arg, and Tyr contents. However, both of them exhibit high Leu and Phe and low Met contents characteristic of other P-450 isoforms reported in the literature [for details, see Black and Coon (1986)]. Further, although not shown here, P-450mt4 exhibits marked differences from another PB-inducible mitochondrial isoform, P-450mt3 (Shayiq & Avadhani, 1989), with respect to amino acid composition, N-terminal sequences, and immunological characteristics. Finally, in order to determine the degree of similarity or divergence between the two immunologically related isoforms, P-450mt4 and P-450b, the N-terminal microsequencing of protein was carried out by the phenylthiohydantoin derivatization method. As shown in Table III, of the 42 residues sequenced, 39 residues show positional identity with P-450b sequences. In positions 32, P-450mt4

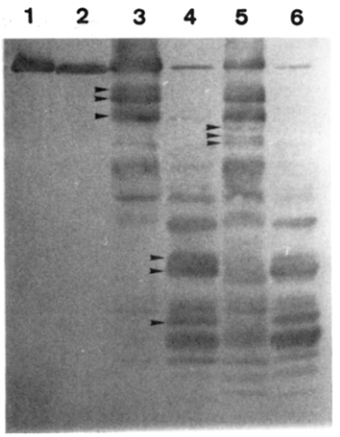

FIGURE 5: V_8 protease fingerprint analysis of P-450mt4 and P-450b. About 15 μg each of P-450b and P-450mt4 was digested with 5 μg of *Staphylococcus aureus* V_8 protease, subjected to electrophoresis on a 16% SDS–polyacrylamide gel, transblotted to a nitrocellulose membrane, and probed with anti-P-450b IgG as described in Figure 2. Lanes 1 and 2 represent 2 μg each of undigested P-450b and P-450mt4, respectively. Lanes 3 and 4 represent P-450b and P-450mt4, respectively, digested for 5 min with protease. Lanes 5 and 6 represent samples as in lanes 3 and 4, digested for 10 min. The arrows in lanes 3–5 indicate the variant peptides obtained with the two P-450 enzymes.

Table II: Amino Acid Compositions of Cytochrome P-450mt4 and P-450b[a]

amino acid	no. of residues/subunit	
	P-450mt4	P-450b[b]
Gly	43	33
Ala	23	23
Val	19	23
Leu	54	63
Ile	24	30
Ser	39	33
Thr	26	27
Cys	*c*	6
Met	3	11
Asn/Asp	37	37
Glu/Gln	50	49
Lys	19	24
Arg	32	31
His	14	17
Phe	31	39
Tyr	13	14
Trp	*c*	1
Pro	23	30
total	450	491

[a] 12–15 pmol of cytochromes P-450 was acid-hydrolyzed, and the amino acid composition was determined by using Applied Biosystems Model 420-A derivatizer/analyzer. [b] P-450b composition taken from Black and Coon (1986). [c] Not estimated.

Table III: N-Terminal Amino Acid Sequence of Cytochrome P-450mt4 in Comparison with P-450b[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
P-450mt4	Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu (Thr)	Leu (Pro)	Ala	Leu	Leu (Thr)	Val	Gly
P-450b[b]	Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu	Leu	Ala	Leu	Leu	Val	Gly
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
P-450mt4	Phe	Leu	Leu	Leu	Leu	Val	Arg	Gly	His	Pro	Lys	Ser	Arg	Gly
P-450b	Phe	Leu	Leu	Leu	Leu	Val	Arg	Gly	His	Pro	Lys	Ser	Arg	Gly
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
P-450mt4	Asn	Phe	Pro	Lys	Gly	Pro	Arg	Pro	Leu	Pro	Lys	Met	Gly	Asn
P-450b	Asn	Phe	Pro	Pro	Gly	Pro	Arg	Pro	Leu	Pro	Leu	Leu	Gly	Asn

[a] About 40–60 pmol of proteins was sequenced by the phenylthiohydantoin derivatization procedure as described under Experimental Procedures. The recovery of amino acids in various runs was in the range of 40–80%. Residues shown in parentheses are variable amino acids detected in three different sequencing runs. [b] P-450b sequence as reported by Fujii-Kuriyama et al. (1982).

Table IV: In Vitro Reconstitution of Monooxygenase Activity Using Various Substrates[a]

cytochrome P-450	reconstituted with	activity [nmol min^{-1} (nmol of P-450)$^{-1}$] (dimethylamino)-antipyrine demethylase	benzphetamine demethylase	vitamin D_3 25-hydroxylase	aflatoxin B_1 metabolism[b]
P-450mt4	Fdx + Fdr	58.1	69.1	0.08	3.6
P-450mt4	cyt P-450 reductase	0.0	0.0	0.0	0.0
P-450b	Fdx + Fdr	0.0	0.0	0.0	0.0
P-450b	cyt P-450 reductase	57.7	63.7		3.69

[a] Reconstitutions were carried out in 0.5-mL volumes using 25–35 pmol of P-450 and Fdx + Fdr or NADPH–cytochrome P-450 reductase electron-transfer systems as described under Experimental Procedures. 2.5 μmol of (dimethylamino)antipyrene, 0.5 μmol of benzphetamine, 100 nmol of [^{3}H]AFB_1 (2200 dpm/nmol), and 100 nmol of vitamin D_3 (11 000 dpm/nmol) were used as the substrates. Reactions were carried out at 37 °C, for 10 min for (dimethylamino)antipyrine and benzphetamine metabolism, and 30 min for AFB_1 and vitamin D_3 hydroxylation. Aminoantipyrine and benzphetamine demethylases were measured as described under Experimental Procedures. The vitamin D_3 25-hydroxylase and AFB_1 hydroxylation were assayed by HPLC analysis of metabolites as described by Raza and Avadhani (1988) and Shayiq and Avadhani (1989), respectively. The values represent an average of two separate estimates. [b] Aflatoxin B_1 metabolites included AFM_1, AFQ_1, AFP_1, and three unknown metabolites, U_1, U_2, and U_3 (Shayiq & Avadhani, 1989).

contains Lys instead of Pro; in position 39, P-450mt4 contains Lys instead of Leu, and in position 40, Met, instead of Leu. Thus, while showing a striking similarity with P-450b, the present mitochondrial isoform exhibits subtle differences to qualify as an independent species belonging to the P-450 IIB gene family.

Mitochondrial Location of P-450mt4. The mitochondrial destination of P-450mt4 was verified by using a direct approach of identifying a putative precursor of P-450mt4 in the cytoplasmic poly(A) RNA directed translation products, and in vitro transport of P-450mt4 apoprotein into isolated hepatic mitochondria. As shown in Figure 6, poly(A) RNA from membrane-free cytoplasmic polysomes directs the synthesis of a number of proteins of heterogenous size in a rabbit reticulocyte lysate system (lane 1). In vitro incubation of total translation products with isolated rat liver mitochondria results in the internalization of a number of proteins into a protease-resistant fraction (see lane 3). Immunoprecipitation of total translation products with anti-P-450b IgG yields two major proteins with apparent molecular masses of 53 and 50 kDa (see lane 2). Although not shown, the 50-kDa protein exhibits electrophoretic mobility identical with P-450b (and P-450mt4), suggesting that it may be P-450b apoprotein. Immunoprecipitation of the in vitro incubated and protease-treated mitochondria, on the other hand, yields only the 50-kDa species, reminiscent of mature P-450mt4 (lane 4). The transport of antibody-reactive 50-kDa species is inhibited by valinomycin + K^+, which disrupt the mitochondrial membrane potential, suggesting that the observed transportation into the protease-resistant compartment indeed involves mitochondrial membrane particles. These results together suggest that P-450mt4 is synthesized as a 53-kDa putative precursor and transported into mitochondria in an energy-dependent manner.

Reconstitution of P-450. The ability of P-450mt4 to metabolize physiological substrates like vitamin D_3 and xenobiotics like benzphetamine, (dimethylamino)antipyrine, and

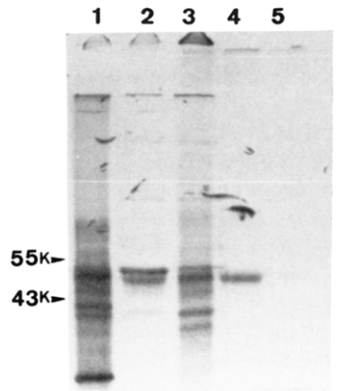

FIGURE 6: Identification of the cytoplasmic precursor of P-450mt4 and its in vitro transport into mitochondria. Poly(A) RNA from cytoplasmic-free polysomes of PB-treated male livers was translated in rabbit reticulocyte lysates in the presence of 1 μCi/μL [^{35}S]-methionine and used for in vitro transport and immunoprecipitation as described under Experimental Procedures. Lane 1, 2 μL (1 × 10^5 cpm) of total translation products; lane 2, 10 μL of translation products immunoprecipitated with anti-P-450b IgG; lane 3, 80 μg of rat liver mitochondria digested with trypsin, following in vitro incubation with 40 μL of translation products; lane 4, a sample as in lane 3 was immunoprecipitated with anti-P-450b IgG following solubilization in an SDS-containing buffer; lane 5, an immunoprecipitate as in lane 4, except that in vitro transport was carried out in the presence of 28 mg/mL valinomycin. The KCl content of the incubation mixture (50 mM) is sufficient to disrupt the membrane potential in the presence of added valinomycin.

aflatoxin B_1 in Fdx + Fdr supported or cytochrome–P-450 reductase supported in vitro systems was studied and compared with the activity of P-450b. As shown in Table IV, P-450mt4 shows high benzphetamine demethylase [58 nmol min^{-1} (nmol of P-450)$^{-1}$], aminoantipyrine demethylase [69 (nmol min^{-1} nmol of P-450)$^{-1}$], and aflatoxin B_1 hydroxylase [3.6 nmol min^{-1} (nmol of P-450)$^{-1}$] activities and relatively low vitamin D_3 25-hydroxylase activity in a Fdx + Fdr supported system.

Further, cytochrome P-450 reductase is unable to support the activity of this enzyme for the metabolism of any of the above substrates. In contrast, P-450b exhibits comparable activities for benzphetamine demethylation [64 nmol min^{-1} (nmol of P-450)$^{-1}$], aminoantipyrine demethylation [58 nmol min^{-1} (nmol of P-450)$^{-1}$], and aflatoxin B_1 activation [3.7 nmol min^{-1} (nmol of P-450)$^{-1}$] and no detectable vitamin D_3 25-hydroxylase activity in a cytochrome P-450 reductase supported system. These results show that although the two P-450 isoforms show comparable activities for three different structurally diverse substrates, they exhibit unique differences with respect to requirement for electron donor systems. The occurrence of mitochondrial-specific electron transport proteins, Fdx and Fdr, in rat hepatic mitochondria has been well documented (Atsuta & Okuda, 1978; Pedersen & Godager, 1978).

Discussion

The characterization of constitutive forms of P-450 from the mitochondrial fractions of various animal tissues like adrenal cortex (Takemore et al., 1975; Hanukoglu et al., 1981), corpus leuteum (Kashiwagi et al., 1980, 1982), kidney (Gazarian et al., 1974; Hitawashi et al., 1982), and liver (Bjorkhem & Gustaffson, 1974; Sato et al., 1977; Pedersen et al., 1979; Niranjan & Avadhani, 1980) has revealed some of the distinctive features of the P-450 isoforms belonging to this cellular compartment. It is generally observed that mitochondrial P-450 isoforms involved in the metabolism of physiological substrates like steroid hormones, bile acids, and vitamin D_3 have a specific requirement for Fdx (an iron–sulfur protein) and Fdr (an iron–sulfur protein reductase) as electron carriers from NADPH [see Sato et al. (1977) and Jefcoate (1986)]. This property of mitochondrial P-450 is reminiscent of P-450 enzymes from the bacterial sources (Katagiri et al., 1968; Sligar & Murray, 1986). Further, the results of cDNA cloning and nucleotide sequence analyses reported for P-450scc and P-45011β (Morohashi et al., 1984; John et al., 1986) show <25% nucleotide and amino acid sequence similarity with those reported for both constitutive and induced forms of microsomal P-450 and a closer similarity to P-450cam. In keeping with this, recent results in our laboratory show no significant immunochemical similarity between the constitutive forms of mitochondrial P-450 and the microsomal isoforms (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989). Thus, because of their distinct catalytic and molecular characteristics and posttranslational processing events, the mitochondrial isoforms have been grouped under a separate family, XI (Nebert et al., 1987; Nebert & Gonzalez, 1987). In the present study, we have purified a hepatic mitochondrial P-450, from PB-induced male rats, which exhibits the catalytic properties and post-translational processing expected of a mitochondrial isoform but shows a close relationship to the microsomal P-450b with respect to immunochemical and molecular properties.

Results of purification and characterization (Figures 1 and 2) show that P-450mt4 is closely similar to P-450b with respect to electrophoretic migration and immunochemical cross-reactivity, although it differs in both respects from other hepatic mitochondrial P-450 isoforms previously purified and characterized in this laboratory. Because of this similarity in size and immunological properties, it was essential to compare the molecular characteristics of the two isoforms in order to rigorously establish the identity of P-450mt4. Further, since some of the isoforms belonging to the IIB gene family such as P-450b and P-450e exhibit up to 97% sequence conservation, a detailed comparison was undertaken using multiple approaches: As shown in Figure 4, P-450mt4 exhibits an apparent isoelectric pH of 6.9 as against a p*I* of 6.5 for P-450b. These results, along with the results of peptide fingerprint analysis presented in Figure 5, demonstrate distinct molecular differences between P-450mt4 and P-450b. These conclusions are further confirmed and extended in experiments on the amino acid composition of the two PB-inducible P-450 isoforms (Table II) showing distinct patterns of similarity and difference with respect to certain amino acids. Furthermore, in support of the results of antibody cross-reactivity presented in Figure 2, the N-terminal sequence analysis shows over 90% sequence conservation between P-450mt4 and P-450b (Table III), and distinct amino acid differences in positions 32, 39, and 40 are observed. The results of the antibody cross-reactivity, together with the amino acid sequence homology, demonstrate that P-450mt4 belongs to the P-450 IIB gene family though it is distinctly different from P-450b and its close relative P-450e.

Thus far, P-450 isoforms belonging to subfamily IIB have been detected only in the hepatic microsomal fraction. Detection of P-450 cross-reactive to anti-P-450b IgG and exhibiting over 90% positional identity in N-terminal amino acid sequences suggested for the first time the occurrence of an isoenzyme belonging to the P-450 IIB gene family in hepatic mitochondria as well. It is now well recognized that proteins destined for the mitochondrial inner membrane and the matrix compartments are translated in the cytoplasm as precursors with 25–70 amino acid long N-terminal extensions, which act as signals for binding to mitochondrial receptor sites and for targeting to the matrix (Hay et al., 1984; Panner & Neupert, 1987). The mitochondrial location of P-450mt4 was verified and confirmed by using an in vitro transport system similar to the one used for the characterization of the anti-P-450c IgG-reactive precursor of P-450mt2 (Niranjan et al., 1988). Results presented in Figure 6 show that translation products programmed with cytoplasmic-free polysomal poly(A) RNA from PB-induced liver contain a significant level of P-450b IgG-reactive 53-kDa protein in addition to the 50-kDa, putative P-450b species. Further, in vitro incubation of translation products results in the internalization of antibody-reactive 50-kDa species with no detectable 53-kDa species. Since proteins belonging to the extramitochondrial compartments generally lack the ability to cross the mitochondrial membrane barrier, it is likely that the anti-P-450b IgG-reactive protein internalized by the mitochondrial membranes is derived from the 53-kDa putative precursor. Thus, our results suggest that P-450mt4 is translated in the cytoplasm as a 53-kDa apoprotein precursor which is transported and processed into the mature 50-kDa protein. The 53-kDa precursor may be analogous to the protein larger than P-450b and P-450e translation products previously detected by Adensnick and Atchison (1986). Recent studies from this laboratory showed that the mitochondrial isoform P-450mt2 (induced by BNF and also 3-methylcholanthrene) is immunologically related to microsomal P-450c (Niranjan et al., 1988; Raza & Avadhani, 1988). Furthermore, a recent report shows a close similarity in size and antibody reactivity between the pyrazole-inducible ethoxycoumarin hydroxylase activities of the microsomal and mitochondrial fractions (Honkakoski et al., 1988). It is therefore likely that there may be a number of inducible types of P-450 isoforms destined for the mitochondrial compartment which might have evolved from genes coding for the microsomal counterparts by gene duplication and recombination. Additional results on cDNA cloning and sequencing are needed to verify this possibility.

As observed for many P-450 isoforms belonging to the IIB gene family, P-450mt4 shows high activities for benzphetamine

demethylation, aminoantipyrine demethylation [58–69 nmol min^{-1} (nmol of P-450)$^{-1}$], and aflatoxin B_1 metabolism [3.6 nmol min^{-1} (nmol of P-450)$^{-1}$]. In addition, it also exhibits a relatively low activity for vitamin D_3 25-hydroxylation. This enzyme, however, shows a characteristic mitochondrial pattern in terms of its specific requirement for Fdx + Fdr as electron carriers (see Table IV). Another interesting observation of the present study is the sex specificity in the induction of P-450mt4. Although some developmental stage dependent variations in the levels of P-450b and P-450e mRNAs have been observed, the overall levels of induction of these microsomal isoforms in both the sexes appear to be the same (Giachelli & Omiecinski, 1986; Nebert & Gunzalez, 1987; also see Figure 3B). Further, developmentally regulated hepatic microsomal enzymes P-4502B and P-450f, two male-specific isoforms (P450g and P-450h), and a female-specific isoenzyme, P-4502d, belonging to subfamily IIA do not show significant immunological cross-reactivity with P-450b (Waxman, 1984). For these reasons, the present PB-inducible isoform appears to be different from the other male-specific isoforms reported in the literature and to our knowledge represents the first sex-specific isoform to be purified from the mitochondrial compartment.

In summary, we describe a male-specific hepatic mitochondrial P-450 which exhibits the properties of a P-450 IIB gene family member as tested by inducibility with PB, N-terminal amino acid sequence similarity, and immunological homology to P-450b. Experiments are under way to determine the physiological importance of this P-450.

ACKNOWLEDGMENTS

We thank Dr. Michael Atchison for a critical evaluation of the manuscript and Doris S. Boyer for helping with the preparation of the manuscript. We are also thankful to Dr. Audree Fowler for helping with the amino acid composition and N-terminal sequence analysis. This paper is dedicated to the fond memories of the late Professor M. Raja Iyengar.

REFERENCES

Adesnik, M., & Atchison, M. (1986) *CRC Crit. Rev. Biochem. 19*, 247–305.

Atchison, M., & Adesnik, M. (1983) *J. Biol. Chem. 258*, 11285–11295.

Atsuta, Y., & Okuda, K. (1978) *J. Biol. Chem. 253*, 4653–4658.

Bhat, N. K., Niranjan, B. G., & Avadhani, N. G. (1982) *Biochemistry 21*, 2452–2460.

Black, S. D., & Coon, M. J. (1986) in *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 161–216, Plenum Press, New York.

Bjorkhem, I., & Gustafsson, J. (1974) *J. Biol. Chem. 249*, 2528–2535.

Bogenhagen, D., & Clayton, D. (1974) *J. Biol. Chem. 249*, 7991–7995.

Burgos-Trinidad, M., Brown, A. J., & Deluca, H. F. (1986) *Biochemistry 25*, 2692–2696.

Cochin, J., & Axelrod, J. (1959) *J. Pharmacol. Exp. Ther. 125*, 105–110.

Conney, A. H. (1982) *Cancer Res. 42*, 4875–4917.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797.

Gelboin, H. V. (1980) *Physiol. Rev. 60*, 1107–1166.

Ghazarian, J. G., Jefcoate, C. R., Knutson, J. C., Orme-Johnson, W. H., & DeLuca, H. F. (1974) *J. Biol. Chem. 249*, 3026–3033.

Giachelli, C. M., & Omiecinski, C. J. (1986) *J. Biol. Chem. 261*, 1359–1363.

Giachelli, C. M., Lin-Jones, J., & Omiecinski, C. (1989) *J. Biol. Chem. 264*, 7046–7063.

Gray, R. W., Omdahl, J. L., Ghazarian, J. G., & DeLuca, H. F. (1972) *J. Biol. Chem. 247*, 7528–7532.

Guengerich, F. P. (1988) *Cancer Res. 48*, 2946–2954.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Keminsky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Hanukoglu, I., & Jefcoat, C. R. (1980) *J. Biol. Chem. 255*, 3057–3061.

Hay, R., Bohini, P., & Gasser, S. (1984) *Biochim. Biophys. Acta 779*, 65–87.

Hitwatashi, A., Nishii, Y., & Ichikawa, Y. (1982) *Biochem. Biophys. Res. Commun. 105*, 329–337.

Jefcoat, C. R. (1977) *J. Biol. Chem. 252*, 8788–8796.

Jefcoate, C. R. (1986) in *Cytochrome P450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 345–386, Plenum Press, New York.

John, M. E., John, M. C., Boggaram, V., Simpson, E. R., & Waterman, M. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4715–4619.

Kashiwagi, K., Dafeldecker, W. P., & Salhanick, H. A. (1980) *J. Biol. Chem. 255*, 2606–2611.

Kashiwagi, K., Carraway, R. E., & Sallianick, H. A. (1982) *Biochem. Biophys. Res. Commun. 105*, 110–116.

Katagari, M., Ganguli, B. N., & Gansalus, I. C. (1968) *J. Biol. Chem. 243*, 3543–3546.

Kimura, H., Sogawa, K., Sakai, Y., & Fujii-Kuriyama, Y. (1989) *J. Biol. Chem. 64*, 2338–2342.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Moos, M., Nguyen, N. Y., & Liu, T. (1988) *J. Biol. Chem. 263*, 6005–6008.

Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S., & Omura, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4647–4651.

Nash, T. (1953) *Biochem. J. 55*, 416–421.

Nebert, D. W., & Gonzalez, F. J. (1987) *Annu. Rev. Biochem. 56*, 945–993.

Nebert, D. W., Adesnik, M., Coon, M. J., Estrabrook, R. W., Gonzalez, F. J., Guengerich, C. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R., & Waterman, M. R. (1987) *DNA (N.Y.) 6*, 1–11.

Niranjan, B. G., & Avadhani, N. G. (1980) *J. Biol. Chem. 255*, 6575–6578.

Niranjan, B. G., Wilson, N. M., Jefcoate, C. R., & Avadhani, N. G. (1984) *J. Biol. Chem. 259*, 12495–12501.

Niranjan, B. J., Raza, H., Shayiq, R. M., Jefcoate, C. R., & Avadhani, N. G. (1988) *J. Biol. Chem. 263*, 575–580.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Pedersen, J. I., & Godager, H. (1978) *Biochim. Biophys. Acta 525*, 28–36.

Pedersen, J. I., Bjorkhem, I., & Gustafsson, J. (1979) *J. Biol. Chem. 254*, 6464–6469.

Pfanner, N., & Neupert, W. (1987) *Curr. Top. Bioenerg. 15*, 177–219.

Rampersaud, A., & Walz, F. G. (1987) *J. Biol. Chem. 262*, 5649–5653.

Raza, H., & Avadhani, N. G. (1988) *J. Biol. Chem. 263*, 9533–9541.

Ryan, D. E., Thomas, P. E., Reik, L. M., & Levin, W. (1982) *Xenobiotica 12*, 727–744.

Sato, R., Atsuta, Y., Imai, Y., Taniguchi, S., & Oleuda, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5477–5481.

Shayiq, R. M., & Avadhani, N. G. (1989) *Biochemistry 28*, 7546–7554.
Simpson, E. R. (1979) *Mol. Cell. Endocrinol. 13*, 213–227.
Sligar, S. G., & Murry, R. I. (1986) in *Cytochrome P450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 429–503, Plenum Press, New York.
Takemori, S. Suhara, K., Hashionoto, S., Hashimoto, M., Sato, H., Gomi, T., & Katagiri, M. (1975) *Biochem. Biophys. Res. Commun. 63*, 588–593.
Takikawa, O., Gomi, T., Suhara, K., Itagaki, E., Takemori, S., & Katagiri, M. (1978) *Arch. Biochem. Biophys. 190*, 300–306.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Waxman, D. (1984) *J. Biol. Chem. 259*, 15481–15490.
Waxman, D. (1986) in *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Motellano, P. R., Ed.) pp 525–539, Plenum Press, New York.
Whitlock, J. (1986) *Annu. Rev. Pharmacol. Toxicol. 26*, 333–369.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.
Yuan, P. M., Ryan, D. E., Levin, W., & Shively, J. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1169–1173.

Cloning and Expression of Three Rabbit Kidney cDNAs Encoding Lauric Acid ω-Hydroxylases[†,‡]

Eric F. Johnson,* Diana L. Walker, and Keith J. Griffin
Division of Biochemistry, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, California 92037
Joan E. Clark, Richard T. Okita, A. Scott Muerhoff, and Bettie Sue Masters
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
Received June 26, 1989; Revised Manuscript Received September 22, 1989

ABSTRACT: cDNAs encoding three cytochrome P-450 enzymes were cloned from a rabbit kidney cDNA library. These three cDNAs exhibit >90% nucleotide sequence identity across the coding region. This degree of sequence identity is also seen with P450IVA4, an enzyme that catalyzes the ω-hydroxylation of prostaglandins and that is elevated during pregnancy and induced by progesterone in rabbit lung. The 3′ untranslated regions of the three cDNAs display very little sequence identity, suggesting that they are the products of distinct genes. The predicted amino acid sequences derived from each cDNA and for P450IVA4 exhibit about 85% identity. Each cDNA was inserted into an expression vector for transient transfection of COS-1 cells. The transfected cells each expressed a protein recognized by antibodies to P450IVA4. Microsomes isolated from the cells transfected with each cDNA efficiently catalyzed the ω-hydroxylation of lauric acid with rates that greatly exceed that catalyzed by microsomes isolated from the host cell line. One of the cDNAs encodes an enzyme that ω-hydroxylates prostaglandin A_1; however, the specific activity was 2 orders of magnitude lower than that for lauric acid. Our results indicate that the substrate selectivity of the kidney P-450s encoded by these cDNAs is distinct from that of the lung P450IVA4 and that multiple enzymes comprise P-450 class IVA in the rabbit.

An unusual aspect of some forms of cytochrome P-450 is their capacity to preferentially oxidize primary rather than secondary or tertiary carbon–hydrogen bonds. This is particularly evident for fatty acid substrates where the hydroxylation of the terminal carbon is referred to as ω-hydroxylation (Kupfer, 1980). The ω-hydroxylated fatty acids are subsequently oxidized further to dicarboxylic acids, which are elevated in man and other species during ketotic states such as starvation or diabetes.

The cytochrome P-450 enzymes that catalyze ω-hydroxylation reactions appear to comprise a distinct family of P-450[1] proteins. A cDNA corresponding to a rat liver lauric acid ω-hydroxylase, P-450 LAω (P450IVA1), has been cloned and characterized (Hardwick et al., 1987; Earnshaw et al., 1988). A comparison of the derived amino acid sequence of the rat P-450 LAω with that of the rabbit lung enzyme (Matsubara et al., 1987), which catalyzes the ω-hydroxylation of prostaglandins E_1, E_2, A_1, A_2, and $F_{2\alpha}$, indicates that they are members of the P450IVA gene family (Nebert et al., 1989). The latter enzyme, termed either P-450 PGω (Williams et al., 1984) or p-2 (Yamamoto et al., 1984), is designated as P450IVA4 in a uniform system of P-450 nomenclature (Nebert et al., 1989).

Rabbit liver and kidney express lauric acid ω-hydroxylases (Lu et al., 1969; Kusunose et al., 1981; Yamamoto et al., 1986; Kusunose et al., 1985), but the kidney enzyme exhibits rela-

[†] Supported by USPHS Grants HD04445 (E.F.J.), GM31296 (B.S.M. and R.T.O.), and ES03771 (R.T.O.). J.E.C. is a Parker B. Francis Fellow in Pulmonary Research.
[‡] The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers M28655-KDB3.seq, M28656-KDA6.seq, and M28657-KDB18.seq.
* Address correspondence to this author at the Research Institute of Scripps Clinic, BCR-7, 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

[1] P-450 is used as a generic term for forms of cytochrome P-450. Rat P-450 LAω and rabbit P-450 p-2 or PGω are designated as P450IVA1 and P450IVA4, respectively, in the uniform system of nomenclature (Nebert et al., 1989). This nomenclature is used for other forms of P-450 discussed in the text. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography.

0006-2960/90/0429-873$02.50/0