Purification and Characterization of a Hepatic Mitochondrial Cytochrome P-450 Active in Aflatoxin B_1 Metabolism†

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ABSTRACT: We have previously shown that phenobarbital (PB) increases hepatic mitochondrial cytochrome P-450 (P-450) content and also the ability to metabolize hepatocarcinogen, aflatoxin B_1 [Niranjan, B. G., Wilson, N. M., Jefcoate, C. R., & Avadhani, N. G. (1984) *J. Biol. Chem. 259*, 12495–12501]. In the present study, we have purified a mitochondrial-specific P-450 with an apparent molecular mass of 52 kdaltons (termed P-450mt3) from PB-induced rat liver using a combination of hydrophobic and ion exchange column chromatography procedures. Polyclonal antibody to P-450mt3 failed to cross-react with P-450mt1 and P-450mt2 purified from β-naphthoflavone- (BNF) induced rat liver mitochondria. Furthermore, P-450mt3 shows an N-terminal amino acid sequence (Ala-Ile-Pro-Ala-Ala-Leu-Arg-Thr-Asp) different from those of both P-450mt1 and P-450mt2, as well as microsomal P-450b. The polyclonal antibody to P-450mt3 cross-reacted with a P-450 of comparable size purified from uninduced mitochondria. These two isoforms, however, showed difference with respect to catalytic properties and amino acid composition. In vitro reconstitution experiments show that P-450mt3 can actively metabolize diverse substrates including (dimethylamino)antipyrine, benzphetamine, and aflatoxin B_1 but shows a low vitamin D_3 25-hydroxylase activity. The mitochondrial P-450 from uninduced livers, on the other hand, shows relatively high [229 pmol min^{-1} (nmol of P-450)$^{-1}$] vitamin D_3 25-hydroxylase activity but a considerably lower ability for aflatoxin B1 metabolism and no detectable activity for (dimethylamino)antipyrine and benzphetamine metabolism. In an in vitro reconstituted assay system, P-450mt3 showed a 2-fold higher aflatoxin B_1–DNA binding activity [2150 pmol (nmol of P-450)$^{-1}$ h^{-1}] than the major PB-inducible microsomal P-450b [1080 pmol (nmol of P-450)$^{-1}$ h^{-1}]. Further, P-450mt3 shows an exclusive requirement for mitochondrial-specific ferredoxin and ferredoxin reductase as carriers of electrons from NADPH. Thus, on the basis of the immunological characteristics, N-terminal amino acid sequence properties, and substrate specificity, P-450mt3 is distinctly different from the two BNF-induced forms reported recently from this laboratory. Further, despite immunological relatedness, P-450mt3 appears to be different from P-450 purified from uninduced hepatic mitochondria.

Cytochrome P-450, an important hemoprotein, functions as the terminal component of the mixed-function oxidase system in both prokaryotic and eukaryotic cells (Gelboin, 1980; Ryan et al., 1979; Lu & West, 1980; Kasper & Gonzalez, 1982). By means of various biochemical and immunochemical techniques, mammalian liver and other tissues have been shown to express multiple forms of P-450[1] capable of catalizing the oxidation and reduction of structurally diverse xenobiotics and physiologically important lipids as well [for reviews, see Coon et al. (1980), Conney (1982), Adesnik and Atchison (1986), Waxman (1986), Whitlock (1986), Waterman (1986), and Jefcoate (1986)]. On the basis of their inducibility with known cytochrome P-450 inducers (Guengerich et al., 1982b; Ryan et al., 1982), their substrate specificities, and their tissue and subcellular locations (Waterman et al., 1986; Jefcoate, 1986), nearly 100 different isoforms have been classified into different families and subfamilies (Black & Coon, 1986; Nebert & Gonzalez, 1987). Phenobarbital is known to induce a number of different cytochromes P-450 which are associated with the hepatic microsomes (Guengerich et al., 1982a,b; Ryan et al., 1982; Nerbert & Gonzalez, 1987; Guengerich, 1988), of which two are sex-dependent types (Waxman, 1984, 1986; Anderson & Jornvall, 1986; Kamataki et al., 1986). There is considerable sequence and immunochemical homology among the members of the same cytochrome P-450 family. For example, cytochrome P-450b (the major PB-inducible type) and cytochrome P-450e belonging to the same subfamily IIB share 97% sequence homology (Yuan et al., 1983; Fujii-Kuriyama et al., 1982; Mizukami et al., 1983). Nevertheless, there is only 20–30% sequence conservation among P-450 beloning to different families.

Except for the adrenal mitochondrial cytochromes P-450scc and P-45011β, which are marginally induced by adrenal corticotropic hormone (DuBois et al., 1981; Kramer et al., 1984; John et al., 1984), studies of cytochrome P-450 induction by xenobiotics and physiologic compounds have been largely focused on the microsomal fraction of different animal cells and tissues [for details, see Waxman (1986), Whitlock (1986), Guengerich (1988), and Nebert and Gonzalez (1987)]. A recent study from our laboratory reported the purification and in vitro reconstitution of two distinct forms of cytochrome P-450 from BNF-induced rat liver mitochondria (Raza &

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[1] Abbreviations: BNF, β-naphthoflavone; PB, phenobarbital; AFB_1, aflatoxin B_1; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; PEG, poly(ethylene glycol) (molecular mass 8000 daltons); kDa, kilodaltons; BaP, benzo[*a*]pyrene; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetraacetate; Adx, adrenodoxin; Adr, adrenodoxin reductase; Fdx, hepatic mitochondrial ferredoxin; Fdr, hepatic mitochondrial ferredoxin reductase; P-450, cytochrome P-450.

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Avadhani, 1988). Earlier reports from our laboratory also showed that mitochondria from PB-treated rat livers contain increased levels of cytochrome P-450 which could be solubilized and reconstituted to metabolize AFB_1 into electrophilic reactive forms (Niranjan & Avadhani, 1980; Niranjan et al., 1984). In the present study we have purified a cytochrome P-450 from PB-induced rat hepatic mitochondria which can activate AFB_1 into DNA binding metabolites in an in vitro system reconstituted with mitochondrial-specific Fdx + Fdr as electron carrier proteins. This enzyme exhibits molecular and catalytic properties different from those of the two isoforms previously purified from BNF-induced rat hepatic mitochondria, as well as the major microsomal forms induced by PB.

Experimental Procedures

Materials. Phenobarbital (sodium salt) was from J. T. Baker Chemical Co. (Phillipsburg, NJ). AFB_1 and its derivatives such as aflatoxins M_1, Q_1, and P_1, 4-(dimethylamino)antipyrine, ω-octylamine–agarose, sodium cholate, PEG, NADP, phosphatidylcholine, and Lubrol PX were purchased from Sigma Chemical Co. (St Louis, MO). DEAE-Sephacel was from Pharmacia Fine Chemicals (Freehold, NJ). Hydroxylapatite (Bio-Gel HTP), Bio-Beads SM2 (20–50 mesh), and chemicals for SDS–polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose membrane for the Western blot analysis was from Schleicher & Schuell Corp. (Keene, NH). Reagents for the Western blot analysis were purchased from Promega Biotech Corp. (Madison, WI). HPLC-grade water, methanol, 2-propanol, and hexane were supplied by Fisher Scientific Co. (King of Prussia, PA). [^{3}H]AFB_1 (16 Ci/mmol) was from Moravek Biochemicals (Breer, CA). [^{3}H]Vitamin D_3 (35–60 Ci/mmol) and [^{3}H]-25-hydroxyvitamin D_3 (150–170 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). All other reagents were obtained from Fluka Chemical Corp. (Ronkonkoma, NY), Sigma Chemical Co. (St. Louis, MO), and Fisher Scientific Co (Pittsburgh, PA).

Isolation of Subcellular Fractions. Male Sprague-Dawley rats (125–150 g) were injected intraperitoneally with PB (70 mg kg^{-1} day^{-1}) for 5 days. The animals were fasted overnight after the last injection and killed by decapitation with a guillotine; the livers were removed, perfused with 0.9% ice-cold NaCl, and used for the preparation of mitochondria and microsomes. The preparation of digitonin-treated mitoplasts and isolation of microsomes were as described before (Bhat et al., 1982b; Niranjan et al., 1984). The resultant mitoplast preparations containing less than 0.6% microsomal-specific NADPH-cytochrome *c* reductase (rotenone insensitive) and glucose-6-phosphatase were used for the purification of cytochrome P-450.

Purification of Mitochondrial Cytochrome P-450. P-450 from PB-induced mitoplast was solubilized by a combination of sonic disruption and cholate treatment and enriched by PEG fractionation (Raza & Avadhani, 1988). P-450mt3 was purified by the method of Guengerich and Martin (1980) using ω-octylamine–agarose, DEAE-Sephacel, and hydroxylapatite chromatography with certain modifications recently described for the purification of two P-450 isoforms from BNF-induced rat hepatic mitochondria (Raza & Avadhani, 1988). The procedure used for partial purification of P-450 from control uninduced rat hepatic mitochondria (Raza & Avadhani, 1988) was improvised on the basis of the extreme lability and the hydrophobic property of this enzyme: The ω-octylamine–agarose column was eluted with a buffer containing 0.4% Lubrol PX to improve the recovery of P-450, and the buffers used for the elution of DEAE and hydroxylapatite columns contained 0.2% Lubrol PX facilitating purification. Further, the purification was speeded up by reducing the length of column chromatography and dialysis steps to minimize the loss of heme. Purification of P-450b from PB-induced rat hepatic microsomes was carried out according to the methods described in the literature (Guengerich & Martin, 1980; Wilson et al., 1984).

Purification of Microsomal and Mitochondrial Electron-Transfer Proteins. NADPH-cytochrome P-450 reductase was purified from PB-induced rat liver microsomes by affinity binding to 2′,5′-ADP–agarose as described by Yasukochi and Masters (1976). The purified NADPH-cytochrome P-450 reductase used in this study was >90% homogeneous (relative molecular mass of 76 kDa) with a specific activity of about 50–60 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$. Fdx and Fdr were purified to near homogeneity (>90%) from canine liver mitochondria following the procedure of Foster and Wilson (1975). Fdx, a 12.4-kDa protein, exhibits an A_{415}:A_{280} ratio of 0.85. Fdr from canine liver is a 56-kDa protein with a specific activity of 13057 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$. Adx and Adr used in this study were a generous gift from Dr. Larry Vickery, University of California at Irvine. Antibodies to various purified proteins were raised in rabbits and made monospecific by affinity adsorption to antigens immobilized on nitrocellulose membrane.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Proteins were subjected to electrophoresis on 12% SDS–polyacrylamide gels and visualized by staining with Coomassie blue (Laemmli, 1970). The conditions of Western blot analysis (Towbin et al., 1979) and immunodetection of protein bands were as described before (Niranjan et al., 1988). Epoxide hydrolase was purified from PB-induced rat liver microsomes following the procedure of Guengerich et al. (1980). The preparation used in this study was about 70% pure with respect to the major 50-kDa protein.

Enzyme Reconstitution and Analysis of Metabolites. Purified mitochondrial or microsomal cytochrome P-450 was reconstituted with NADPH-cytochrome P-450 reductase, Adx + Adr, or Fdx + Fdr electron-transfer systems essentially as described earlier (Niranjan et al., 1984, 1988). Unless otherwise mentioned, the reactions were carried out in 0.5-mL volumes at 37 °C for 10–30 min in a buffer system containing 0.08 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$, 0.2 mM EDTA, 2 mM NADP, 17 mM isocitrate, 80 μg/mL isocitrate dehydrogenase (4.4 units/mg of protein), 20 μg of dilauroylphosphatidylcholine, purified cytochrome P-450 (60–100 pmol/mL), and Fdx (0.4 nmol/mL) + Fdr (0.05 nmol/mL) or NADPH-cytochrome P-450 reductase (0.1 nmol/mL). The mixture was preincubated at 37 °C for 5 min, and the reaction was initiated by addition of appropriate unlabeled or radiolabeled substrates as specified in the individual experiments. In some experiments, Adx (0.4 nmol/mL) and Adr (0.05 nmol/mL) were used for reconstitution of P-450 activity for comparison. (Dimethylamino)antipyrine *N*-demethylase and benzphetamine demethylase activities were measured spectrophotometrically as described by Nash (1953) and Cochin and Axelrod (1959).

In assays using [^{3}H]AFB1 as a substrate, the reaction was stopped by addition of 3 mL of $CHCl_3$. The organic phase containing the metabolites was dried under N_2, taken in methanol, and analyzed by HPLC on a 0.39 × 30 cm Bondapak C18 column (Waters Associates, Milford, MA). The column was developed with an isocratic solvent system con-

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

fractions	total protein (mg)	P-450 (nmol)	specific content [nmol of P-450 (mg of protein)$^{-1}$]	x-fold purification	yield (%)
mitoplast	3240	660	0.203		
15% PEG fraction	1200	370	0.308	1.5	55.8
ω-octylamine–agarose (fractions 42–56)	199	195	0.97	4.7	30.0
DEAE-Sephacel (fractions 40–46)	14	93	6.67	32.8	14.0
hydroxylapatite (fractions 22–30)	1.15	18	15.65	77.09	2.7

taining 50% methanol in 0.05% CH_3COOH at a 0.5 mL/min flow rate, and the fluorescence response at Ex_{365}, Em_{450} was recorded on a Hitachi F1000 fluorescence spectrophotometer. The metabolites were identified by comparison with the retention profiles of known standards, such as AFB_1, AFM_1, AFQ_1, and AFP_1. Individual metabolites were quantitated by comparing the peak areas with the total area of the chromatogram with the help of a Hatachi D-2000 automated integrator system. The values obtained with the fluorescence spectrophotometry were further ascertained by radiometric quantitation of the peaks with a flow-through scintillation counting system, RAMONA (IN/US Corp.), as described before (Raza & Avadhani, 1988). Vitamin D_3 25-hydroxylase was assayed by the two-step HPLC method of Burgos-Trinidad et al. (1986), which involves a straight-phase analysis followed by a reverse-phase separation. The straight-phase analysis was carried out on a 4.6 mm × 25 cm Zorbox-sil column (Du Pont Instruments, Wilmington, DE) in a solvent system of 7% propanol in hexane, and the reverse-phase separation was carried out on the Bondapak C18 column mentioned above in an isocratic solvent system of 85% methanol. The procedures of extraction, HPLC analysis, and quantitation of [^{3}H]vitamin D_3 metabolites were described recently (Raza & Avadhani, 1988).

In Vitro Binding of AFB_1 to DNA. The P-450-dependent formation of DNA binding AFB_1 metabolites was assayed by an in vitro system essentially as described before (Niranjan & Avadhani, 1980; Nirajan et al., 1984). NADPH was generated in 1-mL reaction volumes as described above for the reconstitution of monooxygenase activity. Calf thymus DNA (0.4 mg), [^{3}H]AFB_1 (200 nmol, sp act. 30 μCi/mol), purified P-450 (100 pmol), and Fdx + Fdr or NADPH-cytochrome P-450 reductase in concentrations as described above were added, and the reaction was carried out for 1 h at 37 °C. In some experiments, epoxide hydrolase from PB-induced rat liver microsomes (10 μg) was added. The reaction was stopped by adding SDS at a final concentration of 0.5%, and the unincorporated metabolites and the parent AFB_1 were extracted with $CHCl_3$ (Niranjan & Vadhani, 1980) for HPLC analysis. DNA was isolated from the aqueous phase by extraction with phenol–chloroform followed by ethanol precipitation, and the covalently bound radioactivity was determined as described before (Nirajan & Avadhani, 1980; Niranjan et al., 1984).

N-Terminal Sequence Analysis. About 70–90 pmol of P-450 proteins electrophoretically transferred onto PVDF immobilon membrane (Millipore Corp.) was sequenced in an Applied Biosystems 475A gas-phase sequencer (American BioScience Corp.) 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.

Other Methods. Cytochrome P-450 content was measured according to Omura and Sato (1964) with a 91 cm^{-1} mM^{-1} extinction coefficient for sodium dithionate reduced CO-bound P-450. Protein was measured by the method of Lowry et al. (1951).

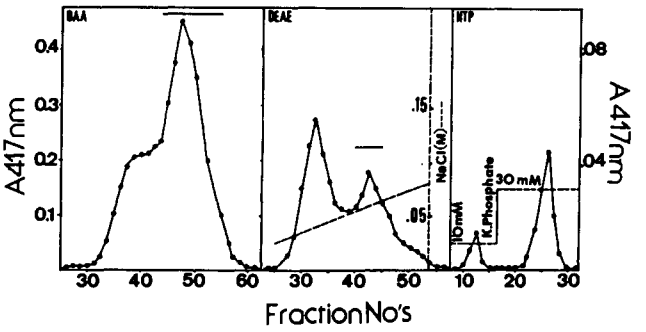

FIGURE 1: Purification of mitochondrial P-450 from PB-induced liver by hydrophobic and ion exchange column chroamtography. Mitochondrial 15% PEG fraction (about 1.2 g of protein) containing about 370 nmol of P-450 as shown in Table I was used for the purification of P-450mt3 by chromatography on ω-octylamine–agarose (OAA), DEAE-Sephacel (DEAE), and hydroxylapatite (HTP) columns. Fractions 42–55 from the OAA column (indicated with a solid line at the top) rich in 417 nm absorbing heme proteins were pooled, concentrated, and applied to the DEAE column. Fractions 40–47 from the DEAE column (indicated with a solid line) were pooled, concentrated, and applied to the HTP column. The amount of protein per P-450 loaded on each column and the recovery have been indicated in Table I.

Results

Purification of P-450 from PB-Induced Mitoplasts. As in our previous studies (Niranjan et al., 1984; Raza & Avadhani, 1988), digitonin-treated, well-washed mitoplasts with less than 0.6% cross-contaminating microsomal fragments were used for the purification of P-450 from PB-induced rat liver. As indicated in Table I, the P-450 was solubilized by a combination of sonication and cholate treatment, enriched by PEG fractionation, and purified by chromatographic separation on ω-octylamine–agarose, DEAE-Sephacel, and hydroxylapatite columns. The elution patterns of heme proteins from ω-octylamine–agarose (OAA), DEAE-Sephacel (DEAE), and hydroxylapatite (HTP) columns have been presented in Figure 1. When the 417 nm absorbing material (fractions 42–55) from the ω-octylamine–agarose column was loaded on to a DEAE-Sephacel column and eluted with a linear gradient of 0–250 mM NaCl, two peaks of heme proteins, one at about 40 mM and the other at about 65 mM NaCl, are eluted (see Figure 1, DEAE). Since peak 1 (fractions 30–35) exhibits a very low specific P-450 content, these fractions were not processed further for purification. DEAE fractions 40–47 rich in P-450 (corresponding to peak 2) were pooled and loaded on to a hydroxylapatite column and eluted with a step gradient of (10, 30, 60, and 90 mM) potassium phosphate buffer. Under these conditions, over 80% of the 417 nm absorbing material from PB-induced mitoplasts is eluted at 10 and 30 mM phosphate buffer (see Figure 1, HTP). The minor peak eluting at 10 mM phosphate appears to be non P-450 heme protein as it failed to show a significant CO-bound P-450 spectrum while the major peak at 30 mM phosphate shows

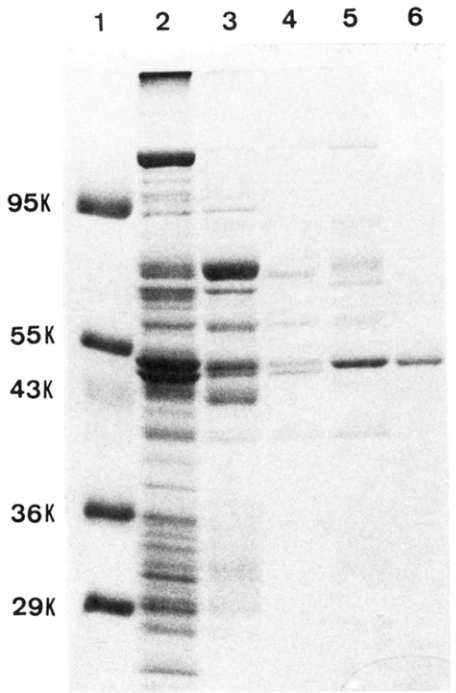

FIGURE 2: Electrophoretic patterns of proteins at different stages of purification. Protein samples were dissociated in a sample buffer containing 4% SDS and 5% 2-mercaptoethanol in a boiling-water bath for 3 min and subjected to electrophoresis on a 12% SDS containing polyacrylamide gel (Laemmli, 1970). Lane 1: molecular mass markers with apparent molecular masses of 95, 55, 43, 36, and 29 kDa. Lane 2: 80 μg of 15% PEG fraction. Lane 3: 25 μg of protein eluted from the OAA column (fractions 42–55, Figure 1). Lane 4: 10 μg of protein from fractions 30–35 of the DEAE column. Lane 5: 10 μg of protein from fractions 40–47 of the DEAE column. Lane 6: 1.5 μg of protein from fractions 22–30 of the HTP column.

a high P-450 content of 15.6 nmol/mg of protein. As shown in Table I, this purification scheme yields about 18 nmol of P-450 (1.15 mg of protein) starting with 3 g of mitoplasts (>600 nmol of P-450), resulting in a near 77-fold purification.

The electrophoretic profiles of P-450 at different stages of purification have been presented in Figure 2. The PEG fraction shown in lane 2 contains major protein species in the range of 50–52 kDa. These species are also seen in fractions eluted from the ω-octylamine–agarose column (see lane 3). Further, peak 1 of the DEAE column (fractions 30–35) exhibiting low P-450 content shows a number of proteins in addition to the two species of 50–52 kDa (see lane 4) corresponding to the major bands seen in the PEG fraction. The second peak of the DEAE column eluting at about 65 mM NaCl, on the other hand, contains a major protein of about 52 kDa. An additional chromatographic separation on hydroxylapatite yields nearly 90% pure protein (see lane 6). Although not shown, this protein exhibits a CO-bound spectrum with an absorption maximum at 448 nm characteristic of P-450. For the sake of presentation the 52-kDa species isolated from PB-induced mitoplasts is referred to as P-450mt3.

Characterization of P-450mt3. The mitochondrial P-450mt3 was compared with similarly induced microsomal P-450b and mitochondrial P-450mt1 and P-450mt2 isolated from BNF-induced mitochondria (Raza & Avadhani, 1988), as well as P-450 purified from uninduced mitochondria (control mt P-450), with respect to electrophoretic migration on SDS–polyacrylamide gels. As shown in Figure 3A (lanes 1 and 2) P-450mt3 exhibits a migration pattern consistent with a molecular mass of about 52 kDa, while microsomal P-450b exhibits a faster migration with a molecular mass of about 50 kDa. The molecular mass estimate for P-450b in this study

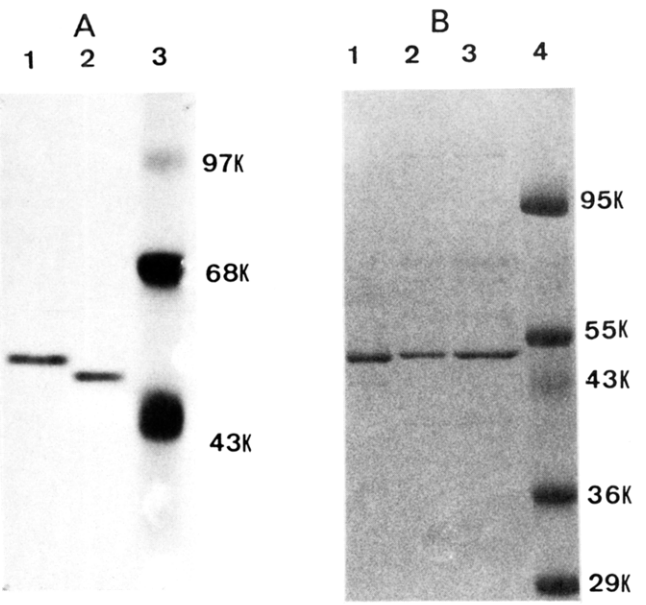

FIGURE 3: Electrophoretic comparison of P-450mt3 with various mitochondrial and microsomal cytochromes P-450. A total of 1.5–2.5 μg of proteins was electrophoresed on 12% polyacrylamide gels containing SDS as described under Experimental Procedures and in Figure 2. (A) P-450mt3 (lane 1) was compared with major PB-inducible microsomal P-450b (lane 2). (B) P-450mt3 (lane 1), BNF-induced P-450mt1 (lane 2), and control mitochondrial P-450 (lane 3) were subjected to electrophoresis on a 12% gel. In lane 3 of (A) and lane 4 of (B), molecular mass markers of the indicated size were run.

is in agreement with the values reported for this isoform by other workers (Guengerich et al., 1982a; Ryan et al., 1982; Black & Coon, 1986). It is also seen from Figure 3B, lanes 1 and 2, that P-450mt3 (lane 1) shows an electrophoretic migration comparable to those of P-450mt1 (lane 2) and control mt P-450 (lane 3) and exhibits an apparent molecular mass of 52 kDa. Further, control mt P-450 isolated in the present study exhibits >70% purity. Although not shown here, P-450mt2 purified from BNF-treated rat liver is considerably larger in size (about 54 kDa) than P-450mt1 (Raza & Avadhani, 1988).

The immunochemical relatedness between P-450mt3 and P-450b as well as other mitochondrial P-450 isoforms was studied by Western blot analysis. As shown in Figure 4A, antibody to P-450b, a major microsomal PB-induced form, does not cross-react with mitochondrial P-450mt3 (lane 2) as well as with other mitochondrial P-450 isoforms such as P-450mt1 (lane 3), P-450mt2 (lane 4), and control mt P-450 (lane 5), although it reacts well with the homologous antigen shown in lane 1. Similarly, antibody to P-450c, a major BNF-induced microsomal form, reacts well with mitochondrial P-450mt2 as shown before (Raza & Avadhani, 1988; Niranjan et al., 1988), though it fails to react with any of the other miotochondrial isoforms (results not presented). These results demonstrate that P-450mt3 is different from the similarly induced microsomal P-450b. In another series of Western blot analysis, PEG fractions from uninduced mitochondria and also PB-induced mitochondria were probed with antibody to purified P-450mt3. As shown in Figure 4B, antibody to P-450mt3 cross-reacts with a minor protein band of about 52 kDa in uninduced mitochondrial PEG (lane 1) and a major band of similar size in the PEG fraction from PB-induced mitochondria (lane 2). Further, this antibody cross-reacts with purified P-450mt3 as well as with control mt P-450 (lanes 3 and 5) but fails to react with P-450mt1 as well as P-450mt2 (lanes 4 and 6) from BNF-induced mitochondria. Similarly,

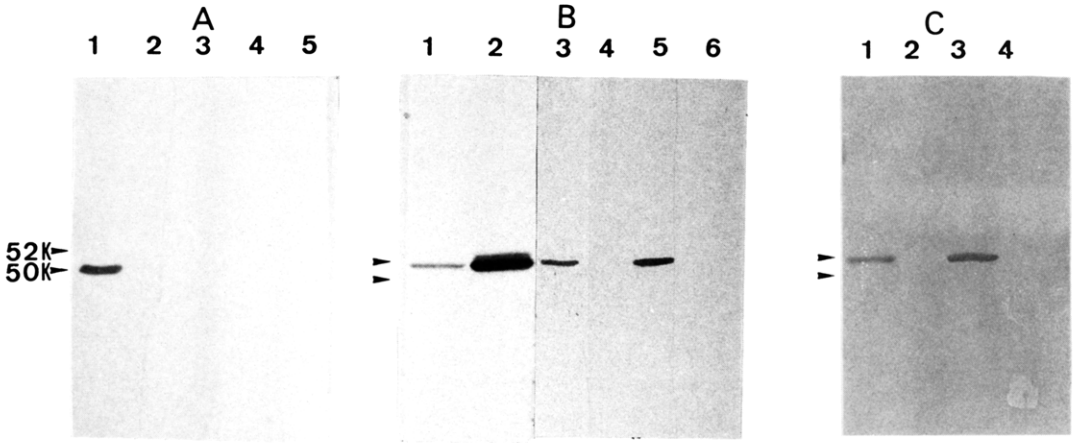

FIGURE 4: Western blot analysis of mitochondrial P-450 and PEG fractions. About 1.5–2.5 μg of purified P-450 proteins and 30–35 μg of 15% PEG fractions were subjected to electrophoresis on 12% SDS–polyacrylamide gels, transblotted to nitrocellulose membranes, and probed with the primary antibodies and alkaline phosphatase conjugated secondary antibodies as described under Experimental Procedures. (A) Lane 1, P-450b; lane 2, P-450mt3; lane 3, P-450mt1; lane 4, P-450mt2; lane 5, control mitochondrial P-450. (B) Lane 1, PEG fraction from control mitochondria; lane 2, PEG fraction from PB-induced mitochondria; lane 3, P-450mt3; lane 4, P-450mt1; lane 5, control mitochondrial P-450; lane 6, P-450mt2. (C) Lane 1, P-450mt3; lane 2, P-450mt1; lane 3, control mitochondrial P-450; lane 4, P-450mt2. Lanes in (A) were probed with a polyclonal antibody against P-450b (dilution 1:1000), lanes in (B) were probed with a polyclonal antibody against P-450mt3 (dilution 1:1000), and lanes in (C) were probed with a polyclonal antibody against control mitochondrial P-450 (dilution 1:1000).

Table II: NH_2-Terminal Amino Acid Sequences of Cytochromes P-450mt1 and P-450mt3[a]

	1	2	3	4	5	6	7	8	9
cytochrome P-450mt1	Ala	Ile	Gly	Ala	Thr	Leu	Thr	Asp	Leu
	(65)	(72)	(60)	(52)	(50)	(58)	(35)	(38)	(30)
cytochrome P-450mt3	Ala	Ile	Pro	Ala	Ala	Leu	Arg	Thr	Asp
	(85)	(52)	(61)	(59)	(56)	(45)	(41)	(44)	(36)

[a] About 70 pmol of P-450mt1 and 90 pmol of P-450mt3, electrophoretically transferred to PVDF membrane, were sequenced by the phenylthiohydantoin derivatization procedure as described under Experimental Procedures. The recovery of amino acids in each cycle ranged from 30 to 85% as indicated in parentheses.

antibody to control mt P-450 cross-reacts with the homologous antigen (lane 1) and also with P-450mt3 (lane 3) but not with P-450mt1 (lane 2) and P-450mt2 (lane 4). These results demonstrate that P-450mt3 is immunologically different from both of the isoforms purified from BNF-induced mitochondria, while it may be closely related to the control mt P-450.

The molecular characteristics of P-450mt3 and BNF-induced P-450mt1 were further compared by N-terminal sequence analysis as shown in Table II. It is seen that the BNF-inducible P-450mt1 exhibits an N-terminal sequence of Ala-Ile-Gly-Ala-Thr-Leu-Thr-Asp-Leu while P-450mt3 contains a sequence of Ala-Ile-Pro-Ala-Ala-Leu-Arg-Thr-Asp, demonstrating distinct differences between the two isoforms which show nearly identical electrophoretic migration. It should also be noted that the N-terminal sequences of these hepatic mitochondrial P-450 isoforms differ from those of other mitochondrial and microsomal P-450 isoenzymes reported in the literature (Black & Coon, 1986). In three separate attempts using up to 200 pmol of protein, however, we have been unsuccessful in determining the N-terminal amino acid sequence of control mt P-450, possibly because for some unknown reasons the amino teminus of this enzyme is oxidatively deaminated during purification.

The amino acid composition of various P-450 isoforms presented in Table III, however, shows distinct differences between P-450mt3 and control mt P-450. The differences are apparent with respect to their Glu/Gln, Ser, Gly, Pro, Val, and Leu contents. Furthermore, P-450mt3 shows differences from P-450mt1 with respect to Asp/Asn, Glu/Gln, Ser, His, Pro, Tyr, and Leu contents. It is also seen that P-450mt3 contains a total of 68 positive amino acid residues (Arg, Lys, and His) as opposed to 60 for control mt P-450 and 45 for P-450mt1.

Table III: Amino Acid Composition of Mitochondrial Cytochromes P-450[a]

	no. of residues/subunit		
amino acid	P-450mt1	P-450mt3	control mt P-450
Asp/Asn	47	35	38
Glu/Gln	52	42	55
Ser	46	37	46
Gly	55	56	68
His	3	17	13
Arg	29	31	25
Thr	27	25	22
Ala	26	30	27
Pro	22	32	21
Tyr	18	9	10
Val	24	30	21
Met	3	5	7
Ile	21	19	18
Leu	42	53	36
Phe	18	23	20
Lys	23	20	22

[a] A total of 15–25 pmol of P-450 isoforms was acid hydrolyzed, and the amino acid composition was determined on an Applied Biosystems Model 420-A derivetizer/analyzer.

Reconstitution of Monooxygenase Activity. Reconstitution experiments were carried out with substrates such as (dimethylamino)antipyrine, benzphetamine, [^{3}H]vitamin D_3, and [^{3}H]AFB_1 in the presence of Fdx + Fdr, NADPH-cytochrome P-450 reductase, and also Adx + Adr. The concentrations of dog liver Fdx and Fdr for optimal reconstitution were found to be 0.4 and 0.05 μM, respectively. Although not shown, use of even 3 times higher concentrations were not inhibitory as long as the Fdx:Fdr ratio was maintained near 8:1. Results of reconstitution experiments presented in Table IV show that in the presence of mitochondria-specific electron-transfer

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

P-450 isoform	reconstituted with	aminoantipyrine demethylase [nmol (nmol of P-450)$^{-1}$ min^{-1}]	benzphetamine demethylase [nmol (nmol of P-450)$^{-1}$ min^{-1}]	vitamin D_3 25-hydroxylase [pmol (nmol of P-450)$^{-1}$ min^{-1}]
P-450mt3	Fdx + Fdr	55.36	61.5	71
P-450mt3	Adx + Adr	52.61		
P-450mt3	NADPH-cyt P-450 reductase	ND[b]	ND	ND
P-450b	Fdx + Fdr	ND	ND	
P-450b	NADPH-cyt P-450 reductase	54.61	69.3	
P-450mt1	Fdx + Fdr	14.88	17.9	51.3
control mt P-450	Fdx + Fdr	ND	ND	229
control mt P-450	NADPH-cyt P-450 reductase	ND	ND	ND

[a] Assays were run in 0.5-mL volumes with 30 pmol of cytochromes P-450 and various electron-transfer systems as described under Experimental Procedures. The substrates, 1.0 μmol of (dimethylamino)antipyrine, 0.5 μmol of benzphetamine, and 200 nmol of [^{3}H]vitamin D_3 (18 000 dpm/nmol), were added to the reaction mixture. Reactions were carried at 37 °C for 10 min for (dimethylamino)antipyrine and benzphetamine and at 37 °C for 30 min for [^{3}H]vitamin D_3. Aminoantipyrine and benzphetamine demethylases were measured as described under Experimental Procedures. The vitamin D_3 metabolites were extracted in methanol–chloroform–H_2O (6:3:2) and analyzed and quantitated by HPLC as described recently (Raza & Avadhani, 1988) and under Experimental Procedures. Values represent an average of two separate experiments. [b] ND = not detected.

Table V: Metabolism of Aflatoxin B_1 by Purified Cytochromes P-450

cytochrome P-450 isoforms	reconstituted with	nmol of product formed min^{-1} (nmol of P-450)$^{-1}$				
		AFQ_1	AFM_1	AFP_1	unknown	total
P-450mt3	Fdx + Fdr	1.83	1.24	0.24	1.11	4.42
P-450mt3	Adx + Adr	1.98	0.89	0.2	0.81	3.88
P-450mt3	NADPH-cyt P-450 reductase	0	0	0	0	0
P-450b	NADPH-cyt P-450 reductase	1.6	0.98	0.19	0.91	3.76
P-450mt1	Fdx + Fdr	0.62	0.97	0.08	0.29	1.96
control mt P-450	Fdx + Fdr	0.28	0.19	0.1	0.07	0.64

[a] Assays were run in 0.5-mL volumes as described in Table III with 100 nmol of [^{3}H]AFB_1 (2200 dpm/nmol) for 30 min at 37 °C. The AFB_1 metabolites were extracted in $CHCl_3$ and analyzed by HPLC as shown in Figure 5. Various metabolite peaks for each assay mixture were identified and quantitated as described under Experimental Procedures. Values represent an average of two separate estimates.

proteins (Fdx + Fdr or Adx + Adr) P-450mt3 exhibits high levels of aminoantipyrine *N*-demethylase [52.6–55.4 nmol min^{-1} (nmol of P-450)$^{-1}$] and benzphetamine demethylase [61.5 nmol min^{-1} (nmol of P-450)$^{-1}$] activities and relatively low vitamin D_3 25-hydroxylase activity [70 pmol min^{-1} (nmol of P-450)$^{-1}$]. Furthermore, P-450mt3 was unable to metabolize any of the three substrates when reconstituted with microsomal-specific NADPH-cytochrome P-450 reductase (see Table IV). These results demonstrate that, unlike the two mitochondrial isoforms from BNF-induced liver, P-450mt3 has exclusive specificity for mitochondrial-specific electron-transfer systems for activity. In comparison, P-450mt1 from BNF-induced hepatic mitochondria showed a considerably lower activity for aminoantipyrine N-demethylation and benzphetamine demethylation in the range of 14–18 nmol min^{-1} (nmol of P-450)$^{-1}$]. Furthermore, P-450mt1 shows significant vitamin D_3 25-hydroxylase activity as shown in a recent report from this laboratory (Raza & Avadhani, 1988). It is also seen from Table IV that control mt P-450 exhibits a vitamin D_3 25-hydroxylase activity of 229 pmol min^{-1} (nmol of P-450)$^{-1}$ in an Fdx + Fdr supported system. Further in support of results of our own (Raza & Avadhani, 1988) and others (Dahlback & Wikvall, 1988; Masumoto et al., 1988), the activity of control mt P-450 is maximally supported by mitochondrial-specific electron-transfer proteins and minimally with microsomal-specific NADPH-cytochrome P-450 reductase.

Since previous studies from this laboratory showed that mitoplasts or mitochondrial PEG fractions from PB-induced rat liver metabolize AFB_1 into electrophilic reactive forms (Niranjan & Avadhani, 1980; Niranjan et al., 1984, 1986), we tested the ability of purified P-450mt3 to metabolize this hepatocarcinogen in an in vitro reconstituted system. The HPLC pattern presented in Figure 5A shows that AFB_1 incubated in a reconstituted system without added P-450 elutes with a retention time similar to that of parent AFB_1 indicating no detectable metabolism. The elution patterns with added

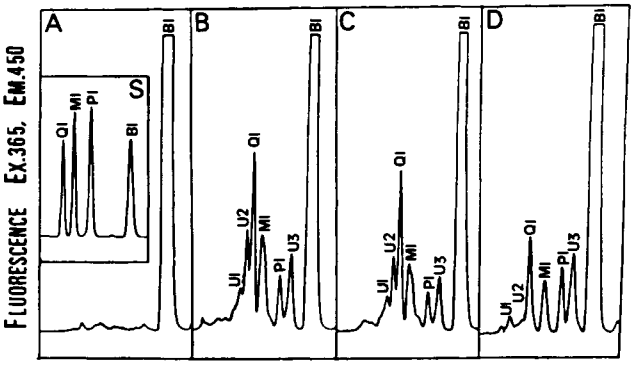

FIGURE 5: HPLC analysis of aflatoxin B_1 metabolites. Aflatoxin B_1 metabolites and also the unmetabolized parent carcinogen were extracted from the in vitro reconstituted reaction mixtures and analyzed on a Bondapak C18 column as described under Experimental Procedures. The profile in (A) represents an in vitro reaction run with Fdx + Fdr and without added P-450, the profile in (B) represents a reaction run with Fdx + Fdr and P-450mt3, the profile in (C) represents a reaction run with NADPH-cytochrome P-450 reductase and P-450b, and the profile in (D) represents a reaction run as in (B) with added calf thymus DNA. The inset (S) indicates the retention profiles of known aflatoxin markers: AFQ_1 (Q_1), AFM_1 (M_1), AFP_1 (P_1), and AFB_1 (B_1). Peaks in panels B–D marked U_1 and U_2 are metabolites possibly related to the DNA-reactive epoxides. The nature of peak U_3 remains unknown.

P-450mt3 (Figure 5B) and P-450b (Figure 5C), on the other hand, show peaks with retention times similar to those of known AFB_1 metabolites like AFM_1, AFQ_1, and AFP_1 and also three unknown metabolites (designated as U_1, U_2, and U_3) in addition to the parent AFB_1 peak. Although not shown, in parallel experiments [^{3}H]AFB_1 was used as the substrate, and the radioactivity pattern of the HPLC fractions was recorded with a flow-through scintillation counter (RAMONA). The quantitation of metabolites was carried out by comparing the area of a given peak with the total area of the chromatogram. The values based on the fluorescence pattern were nearly identical with those calculated on the basis of radio-

Table VI: Cytochrome P-450 Catalyzed Binding of [^{3}H]AFB_1 to Calf Thymus DNA under in Vitro Conditions[a]

cytochrome P-450 isoform	reconstituted with	epoxide hydrolase (μg of protein)	pmol of [^{3}H]AFB_1 bound to DNA (nmol of P-450)$^{-1}$ h^{-1}
none	Fdx + Fdr	0	0
P-450mt3	Fdx + Fdr	0	2150
P-450mt3	Fdx + Fdr	10	100
P-450b	NADPH-cyt P-450 reductase	0	1080
P-450b	NADPH-cyt P-450 reductase	10	45
control mt P-450	Fdx + Fdr	0	65

[a] Assays were run as described under Experimental Procedures with added calf thymus DNA and with or without added epoxide hydrolase as indicated. After 1-h incubation at 37 °C, the DNA was isolated by extraction with phenol–chloroform followed by ethanol precipitation, and covalently bound aflatoxin was quantitated as described before (Niranjan et al., 1984) and under Experimental Procedures. Values represent an average of two separate estimates.

activity determinations. These values for different P-450 isoforms reconstituted with different electron donor systems have been presented in Table V. It is seen that P-450mt3 reconstituted with Fdx + Fdr and Adx + Adr yields AFM_1, AFQ_1, AFP_1, and three unknown metabolites, with total metabolic activities of about 5 and 4 nmol, respectively. In confirmation of results of monooxygenase metabolism presented in Table IV, NADPH-cytochrome P-450 reductase is unable to support the reconstitution of this isoform for AFB_1 metabolism. P-450mt1 and control mt P-450 on the other hand show relatively lower activity of 0.6–2 nmol min^{-1} (nmol of P-450)$^{-1}$ for AFB_1 metabolism. Furthermore, some of the prominent metabolites such as AFQ_1 and AFP_1 occur at drastically reduced levels (see Table V), and also, two of the unknown metabolites, U_1 and U_2, are completely missing (results not presented) in reaction mixtures with added P-450mt1. The results of in vitro reconstitution are in agreement with previous results showing a generally low level of reactive AFB_1 metabolites in incubation mixtures with 3-methylcholanthrene-induced mitoplasts or mitochondrial PEG fractions (Niranjan et al., 1984). These results together demonstrate that P-450mt3 represents a unique hepatic mitochondrial form different from the two BNF-induced forms previously reported.

The relative levels of electrophilic reactive metabolites of AFB_1 generated in the in vitro reconstituted system were determined by a DNA binding method similar to those used for the microsomal (Asao et al., 1965; Gurtoo & Campbell, 1974) and mitochondrial PEG fractions (Niranjan & Avadhani, 1980; Niranjan et al., 1984, 1986). Results presented in Table VI show that the P-450mt3-supported in vitro system is nearly 2-fold more active in the formation of DNA adducts [36 pmol of AFB_1 bound to DNA (nmol of P450)$^{-1}$ min^{-1}] than the P-450b-catalyzed in vitro system [18 pmol (nmol of P-450)$^{-1}$ min^{-1}] and <30-fold more active than the control mt P-450 [about 1 pmol min^{-1} (nmol of P-450)$^{-1}$]. Further, the in vitro AFB_1–DNA binding catalyzed by all of these P-450 isoforms under study is drastically reduced by the addition of epoxide hydrolase, confirming that the in vitro DNA binding is due to the formation of an epoxide (Swenson et al., 1977; Essigmann et al., 1977). As seen from Figure 5D, the HPLC profiles of unbound metabolites show drastically reduced U_1 and U_2 in reaction mixtures with added calf thymus DNA. Further, addition of epoxide hydrolase and resultant inhibition of AFB_1–DNA binding restores the levels of U_1 and U_2, suggesting that these two peaks are the 2,3-epoxide derivatives of various aflatoxins (AFB_1, AFM_1, AFP_1).

Discussion

It is known that hepatic mitochondria can metabolize diverse xenobiotic agents (Niranjan & Avadhani, 1980; Niranjan et al., 1982, 1984, 1985, 1986; Hankakoski et al., 1988) in addition to physiologically important C-27 steroids (Bjorkhem & Gustafsson, 1974). A recent study from this laboratory showed the occurrence of two distinct forms of cytochrome P-450 termed P-450mt1 and P-450mt2 in mitochondria from BNF-induced rat liver (Raza & Avadhani, 1988). Earlier studies also showed that treatment with PB results in a marked increase in mitochondrial P-450 content (Niranjan & Avadhani, 1980; Niranjan et al., 1984) and their ability to metabolize hepatic carcinogen AFB_1 to electrophilic reactive forms which covalently modify endogenous DNA in intact mitoplasts, as well as externally added calf thymus DNA in mitochondrial PEG fractions (Niranjan et al., 1984). In the present study, we have purified a P-450 from PB-induced rat liver mitochondria and compared its molecular and catalytic properties with those of BNF-inducible P-450mt1 and P-450mt2 as well as a P-450 from control hepatic mitochondria to determine if it is an independent isoform.

The low P-450 content of hepatic mitochondria (Niranjan et al., 1984; Bjorkhem & Gustafsson, 1974) presents a considerable problem in the purification, and as indicated in our previous study (Raza & Avadhani, 1988), xenobiotic inducers help increase and stabilize the enzyme, enabling the purification of these mitochondrial isoforms. Using the purification scheme essentially similar to those used for the purification of P-450mt1 and P-450mt2 from BNF-induced mitochondria (Raza & Avadhani, 1988), we have been able to achieve a 77-fold purification of P-450 from PB-induced hepatic mitochondria (termed P-450mt3) with a specific content of 16 nmol/mg. The final yield, however, is only about 2.5–3.0% of the input P-450. Also, using nearly twice the amount of starting material and rapid isolation steps, we have purified a mitochondrial P-450 from uninduced male livers (termed control mt P-450) to about 70% purity. The heme content of this control mt P-450 is only about 4–5 nmol/mg, suggesting its sensitivity to handling and purification steps. As judged by the electrophoretic pattern in Figure 2 (lane 5), the P-450mt3 purified by this method is nearly 90% homogenous and exhibits a molecular mass of about 52.0 kDa. When this paper was being written up, two groups reported the purification of P-450 from uninduced rabbit liver (Dahlback & Wikvall, 1988) and rat liver mitochondria (Masumoto et al., 1988), which resemble control mt P-450 purified in the present study in size and catalytic properties (Figure 3B, lane 3). The Western blot experiments presneted in Figures 4 and 5 suggest the possibility that P-450mt3 differs from P-450mt1 and P-450mt2 as well as from microsomal P-450b. First, antibody to P450b does not significantly cross-react with any of the mitochondrial P-450 isoforms (Figure 4A). Second, P-450mt3 and control mt P-450 share significant immunological homology as they cross-react with the converse antibodies (see Figure 4B,C). Finally, both of these latter antibodies failed to react with P-450mt1 (see Figure 4B,C) as well as with P-450mt2 (Figure 4B,C). These results strongly imply that P-450mt3 is an independent species different from the two BNF-inducible types purified and characterized in our previous study (Raza & Avadhani, 1988).

Despite similarity in size and immunochemical cross-reactivity, P-450mt3 appears to be a distinctly different species from the control mt P-450 purified in the present study for the following reasons: First, the amino acid compositions of

the two isoforms show marked differences, and P-450mt3 is probably the most basic mitochondrial isoform thus far we have encountered (latter results not presented). Second, the in vitro reconstitution experiments presented in Tables IV and V show that P-450mt3 actively catalyzes the demethylation of (dimethylamino)antipyrine and benzphetamine and shows a relatively low vitamin D_3 25-hydroxylation activity. P-450mt1 and control mt P-450 on the other hand show negligible to very low activity for (dimethylamino)antipyrine and benzphetamine demethylation and significant to relatively high levels of vitamin D_3 25-hydroxylation. In agreement with the results of Dahlback and Wikvall (1988) and Masumoto et al. (1988), the control mt P-450 exhibits the properties of mitochondria-specific vitamin D_3 25-hydroxylase. A recent paper from this laboratory (Raza & Avadhani, 1988) reported a low vitamin D_3 25-hydroxylase activity [18 pmol (nmol of P-450)$^{-1}$ min^{-1}] with a partially purified P-450 from uninduced male livers (Raza & Avadhani, 1988). Recent unpublished results attributed this low activity to the fact that the preparation was enriched with a second constitutive form of mitochondrial P-450 active in cholesterol 25- and 26-hydroxylations. Further characterization of this second constitutive isoform is under way.

Another distinctive and perhaps important difference between the three mitochondrial isoenzymes under study relates to the level as well as the pattern of AFB_1 activation. P-450mt3 exhibits 2.5–8-fold higher AFB_1 metabolism than control mt P-450 and P-450mt1 (Table V). It is also seen that P-450mt3 yields nearly 10 times higher levels of polar metabolites labeled as U_1 and U_2 (Figure 5) which appear to be the immediate products of various aflatoxin 2′,3′-epoxides that have been implicated in the AFB_1 hepatocarcinogenesis (Swenson et al., 1977; Essigman et al., 1977). In this respect it is interesting to note that P-450mt3 shows nearly a 2-fold higher activity for AFB_1–DNA binding in an in vitro reconstituted system than the similarly induced microsomal P-450b (see Table VI), while its overall activity for AFB_1 metabolism is only about 20% higher than that of the latter. It is known that AFB_1 administered to experimental animals tends to accumulate in the hepatic mitochondrial fraction (Bhat et al., 1982a; Niranjan et al., 1982) and modifies mitochondrial DNA at frequencies severalfold higher than nuclear DNA (Niranjan et al., 1982, 1986). In our estimate, P-450mt3 is the predominant mitochondrial isoform which occurs at a relative abundance of 0.08–0.1 of P-450b and P-450e in PB-induced liver. These observations suggest the possibility that the mitochondrial compartment may play a significant role in the metabolism and disposition of hepatic carcinogen AFB_1 in the liver.

The results of reconstitution experiments (Tables IV and V) demonstrate that P-450mt3 purified from PB-induced mitochondria has an absolute requirement for mitochondrial-type Fdx + Fdr for catalytic reconstitution, suggesting that it is closely related to the control mitochondrial P-450 purified in this study and also to those characterized from mitochondria from other tissues (Kimura, 1981; Waterman et al., 1986; Jefcoate, 1986). Isolation and characterization of mitochondrial P-450 showing absolute requirement for Fdx + Fdr, and yet resembling the microsomal forms with respect to xenobiotic induction and substrate specificity, raise interesting questions on their evolutionary mode and ancestry. In summary, the present studies provide a rigorous proof for the occurrence of multiple species of P-450 in hepatic mitochondria.

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Registry No. P-450, 9035-51-2; Fdr, 39369-37-4; PB, 50-06-6; AFB_1, 1162-65-8; monooxygenase, 9038-14-6; vitamin D_3 25-hydroxylase, 65589-62-0; (dimethylamino)antipyrine, 58-15-1; benzphetamine, 156-08-1.

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