Hepatic Mitochondrial Cytochrome P-450 System

PURIFICATION AND CHARACTERIZATION OF TWO DISTINCT FORMS OF MITOCHONDRIAL CYTOCHROME P-450 FROM β-NAPHTHOFLAVONE-INDUCED RAT LIVER*

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We have purified two distinct isoforms of mitochondrial cytochrome P-450 from β-naphtoflavone (β-NF)-induced rat liver to >85% homogeneity and characterized their molecular and catalytic properties. One of these isoforms showing an apparent molecular mass of 52 kDa is termed P-450mt1 and the second isoform with 54-kDa molecular mass is termed P-450mt2. Cytochrome P-450mt2 comigrates with similarly induced microsomal P-450c (the major β-NF-inducible form) on sodium dodecyl sulfate-polyacrylamide gels and cross-reacts with polyclonal antibody monospecific for cytochrome P-450c. Cytochrome P-450mt2, however, represents a distinct molecular species since it failed to react with a monoclonal antibody to P-450c and produced V8 protease fingerprints different from P-450c. Cytochrome P-450mt1, on the other hand, did not show any immunochemical homology with P-450c or P-450mt2 as well as partially purified P-450 from control mitochondria. Electrophoretic comparisons and Western blot analysis show that both P-450mt1 and P-450mt2 are induced forms not present in detectable levels in control liver mitochondria.

A distinctive property of mitochondrial P-450mt1 and P-450mt2 was that their catalytic activities could be reconstituted with both NADPH-cytochrome P-450 reductase as well as mitochondrial specific ferredoxin and ferredoxin reductase electron transfer systems, while P-450c showed exclusive requirement for NADPH-cytochrome P-450 reductase. Cytochromes P-450mt1 and P-450mt2 were able to metabolize xenobiotics like benzo(a)pyrene and dimethyl benzanthracene at rates only one-tenth with cytochrome P-450c. Furthermore, P-450mt1, P-450mt2, as well as partially purified P-450 from control liver, but not P-450c, showed varying activities for 25- and 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D₃. These results provide evidence for the presence of at least two distinct forms of β-NF-inducible cytochrome P-450 in rat hepatic mitochondria.

The cytochromes P-450 belong to a family of hemoproteins that catalyze the oxidation and reduction of structurally diverse xenobiotics and endogenous compounds with unique as well as overlapping substrate specificity (1-6). The occurrence of multiple forms of P-450 in the liver (4, 7) and also in some nonhepatic tissues (8, 9) has been ascribed to their ability to metabolize a diverse array of substrates. Various forms of P-450 have been purified and characterized with respect to their molecular and catalytic properties from the microsomes of hepatic and extrahepatic tissues (2, 10, 11). Similarly, two isoforms of P-450 involved in cholesterol side chain cleavage and 11β-hydroxylation of cholesterol have been purified from mitochondria of steroidogenic tissues, like adrenal cortex, and fully characterized (12-14). In addition, a cytochrome P-450 from renal mitochondria involved in 1α-hydroxylation of 25-hydroxy vitamin D₃ has also been purified and characterized with respect to catalytic properties (15, 16).

It is also well established that hepatic mitochondria contain cytochrome P-450 with properties specific for enzymatic activities associated with hepatic mitochondria (19-21). Similarly, a number of reports including our own (22-24) have shown that rat hepatic mitochondria contain a cytochrome P-450-type enzyme system to metabolize various xenobiotics as well. We have reported that the rat liver mitochondrial P-450 is induced by 3-MC and phenobarbital (24). The P-450 solubilized from rat hepatic mitochondria induced with 3-MC and phenobarbital could be reconstituted in vitro in the presence of certain carcinogens like benzo(a)pyrene, aflatoxin, and dimethyl nitrosamine (24). The distinctive property of the mitochondrial P-450 in our study was based on its ability to accept electrons from NADPH through mitochondrial-specific iron-sulfur protein (adrenodoxin) and a flavoprotein (adrenodoxin reductase). Recent experiments in our laboratory also identified a 57-kDa protein in the translation products of mitochondrial iron-sulfur protein (ferredoxin). This protein is able to catalyze 25-hydroxylation of vitamin D₃ and 26-hydroxylation of C-27 sterols (17-20). The pattern of induction by known inducers and inhibition by competing substrates suggest the possibility that separate enzymes may be responsible for the two hydroxylation activities associated with hepatic mitochondria (19-21). Similarly, a number of reports including our own (22-24) have shown that rat hepatic mitochondria contain a cytochrome P-450-type enzyme system to metabolize various xenobiotics as well.

The abbreviations used and trivial names used are: P-450, cytochrome P-450; 3-MC, 3-methylcholanthrene; β-NF or BNF, β-naphtoflavone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PEG, polyethylene glycol (molecular mass, 8000 daltons); BaP, benzo[a]pyrene; DMBA, dimethylbenzanthracene; DTT, dithiothreitol; Adx, adrenodoxin; Adr, adrenodoxin reductase; ISP, iron-sulfur protein reductase (ferredoxin reductase) from mouse liver mitochondria; HPLC, high pressure liquid chromatography.

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ence of adrenodoxin + adrenodoxin reductase and hepatic mitochondrial ferredoxin + ferredoxin reductase. Further, the purified enzymes show properties distinctly different from the similarly induced microsomal P-450c (a major β-NF- or 3-MC-inducible type) with respect to catalytic properties, immunochemical characteristics, and peptide fingerprint patterns.

**MATERIALS AND METHODS**

**RESULTS**

The Pattern of Cytochrome P-450 Induction by β-Naphthoflavone—In our previous studies, 3-MC was shown to induce the rat hepatic mitochondrial cytochrome P-450 content and increase their ability to metabolize (a)pyrene by severalfold (24). We also showed that the antibody to microsomal P-450c detected a 54-kDa protein, a putative mitochondrial-specific P-450 in mitochondria from 3-MC-treated rat liver which could not be detected in sufficient quantity in control liver mitochondria (25). It is well established that 3-MC and β-NF induce nearly identical species of hepatic microsomal P-450 (26). The results of Western blot analysis and immunodetection presented in Fig. 1 (Miniprint) show that the PEG fractions from 3-MC-induced and β-NF-induced rat liver mitochondria contain the 54-kDa protein reactive to polyclonal antibody to microsomal P-450c. Nevertheless, the level of induction with β-NF appears to be significantly higher than with 3-MC (lanes 1 and 2). Furthermore, a protein of similar size can be purified by affinity binding to the anti-P-450 IgG column from PEG fractions of mitochondria induced with both of these compounds (lanes 3 and 4). Although not shown, the PEG fraction from β-NF-induced liver mitoplasts was able to metabolize benzo(a)pyrene in an in vitro system reconstituted with Adx + Adr as well as NADPH-cytochrome P-450 reductase as previously shown for the 3-MC-induced mitochondrial fraction (24). These results therefore suggest that as in the case of the microsomal system, β-NF induces a mitochondrial P-450 species similar to 3-MC. For this reason, β-NF-induced mitochondrial and microsomal fractions were used in all of the subsequent experiments reported in this paper.

**Chromatographic Properties and Recovery of Cytochrome P-450**—Mitochondria stripped out of the outer membrane by digitonin treatment (mitoplasts) from control and β-NF-treated rat liver were used as the source of mitochondrial cytochrome P-450. The cross-contaminating microsomal fraction in these preparations was in the range of 0.4–0.8% as judged by the marker enzyme assays carried out on a routine basis as described under “Materials and Methods.” The cytochrome P-450 was solubilized by a combination of sonic disruption and cholate treatment, enriched by PEG fractionation, and purified in steps using a combination of hydrophobic chromatography on ω-octylamine agarose, ion-exchange chromatography on DEAE-Sephacel, and hydroxylapatite as indicated in Table I. The P-450 solubilized from similarly induced microsomal fraction was used for comparison. In a typical purification procedure, about 3 g of mitoplasts isolated from 40 to 45 rat livers were used as the starting material. As seen from Table I, the P-450 content of the cholate extract from control mitoplast was about 0.07 nmol/mg protein while similar fractions from β-NF-induced mitoplasts showed 0.16–0.2 nmol/mg suggesting a 2.5–3.0-fold induction. The DEAE elution patterns presented in Fig. 2 (Miniprint) show distinct differences between the mitochondrial and microsomal P-450. Over 90% of the input P-450 from control and β-NF-induced mitoplasts is eluted at about 70–90 mM NaCl concentration while under the identical conditions the microsomal P-450 elutes out at 110–130 mM salt. It is also seen that there is a minor difference between the elution profile of control and β-NF-induced mitochondrial P-450 since a fraction of the latter species elutes out heterogeneously ranging to 100 mM salt (see Fig. 2, Miniprint). In addition, a minor species of heme protein of unknown nature elutes at 50 mM salt from β-NF-induced mitochondrial extracts. As shown in Fig. 3, the β-NF-induced mitochondrial P-450 bound to the hydroxylapatite column elutes in two peak fractions at 40 and 60 mM potassium phosphate. The β-NF-induced microsomal P-450 on the other hand elutes at >90 mM salt as previously reported by Guengerich et al. (32). The material eluting at 10 mM potassium phosphate in both cases does not show the CO-reduced spectrum characteristic of P-450 (results not shown), suggesting that it is a non-P-450 heme protein with absorption at 417 nm. Although not shown, >60% of the input cytochrome P-450 from control mitochondria elutes from the hydroxylapatite column at 40 mM potassium phosphate.

The P-450 fractions eluted from the hydroxylapatite column at 40, 60, or >90 mM potassium phosphate containing buffer were further purified by an additional chromatography on hydroxylapatite when needed. As shown in Table I, the resultant cytochrome P-450 from β-NF-induced mitochondria and microsomes exhibited P-450 contents of 13–16 nmol/mg protein. The P-450 content of control mitochondrial isolate, however, remained low (2–3 nmol/mg) at the end of these purification steps.

Characterization of Mitochondrial Cytochromes P-450—The electrophoretic patterns of protein fractions at various stages of purification of P-450 from β-NF-induced mitochondria have been presented in Fig. 4A. It is seen that proteins in the region of about 52–56 kDa become enriched at ω-octylamine agarose and DEAE-Sephacel chromatography steps (lanes 1–3). The hydroxylapatite fractions 31–33 eluting at 40 mM potassium phosphate contain a 52-kDa protein, which shows a CO-reduced spectrum with an absorption maximum at 449.5 nm characteristic of cytochrome P-450 (Fig. 4, A and B). This preparation is >85% pure as seen from the electrophoretic patterns presented in lanes 7–9. The second 417-nm absorbing peak eluting at 60 mM salt (Fig. 3 in Miniprint, fractions 46–49) consists of >90% homogeneous protein with an apparent molecular mass of 54 kDa (see lanes 5 and 6). The electrophoretic pattern of this heme protein is closely similar to that of microsomal P-450c presented in lanes 10 and 11. Further, as seen from Fig. 4B, this protein also exhibits a CO-reduced spectrum characteristic of cytochrome P-450 with an absorption maximum at 449.5 nm. These results suggest the occurrence of two separate species of P-450 in β-NF-induced mitochondria. For the sake of presentation, the faster migrating P-450 with an apparent molecular mass of 52 kDa has been designated as P-450mt1 and the slower migrating 54-kDa species as P-450mt2.

Fig. 5 shows the electrophoretic patterns of protein fractions from control uninduced mitochondria at various stages of P-450 purification. It is seen that a minor species of about 52 kDa from the PEG fraction (lane 1) is only marginally enriched in the ω-octylamine agarose and DEAE-Sephacel steps (lanes 2 and 3). Lanes 4 and 5 represent early and midpeak fractions of 417-nm absorbing material eluted from the hydroxylapatite column at 40 mM potassium phosphate.
Cytochrome P-450 from Hepatic Mitochondria

TABLE I

Cytochromes P-450 contents at various stages of purification

<table>
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<tr>
<th>Fraction</th>
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<td></td>
<td>Protein</td>
<td>P-450</td>
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<td>Protein</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>nmol</td>
<td>nmol/mg</td>
<td>mg</td>
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<td>Cholate extract</td>
<td>6320</td>
<td>8548</td>
<td>1.52</td>
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<td>5–15% PEG fraction</td>
<td>1875</td>
<td>3950</td>
<td>2.10</td>
<td>850</td>
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<tr>
<td>OAA* bound fraction</td>
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<td>1560</td>
<td>5.8</td>
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<tr>
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<td>360</td>
<td>11.25</td>
<td>34</td>
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<tr>
<td>Hydroxylapatite eluate</td>
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<tr>
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<tr>
<td>90 mM phosphate</td>
<td></td>
<td></td>
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</tbody>
</table>

* Specific content of P-450.
* Values represent an average of two separate estimates.
* OAA, ω-octylamine agarose.

FIG. 4. Electrophoretic patterns of cytochrome P-450 from β-NF-induced mitochondria. A, protein fractions from β-NF-induced mitochondria at various stages of purification were subjected to SDS-PAGE and visualized by staining with Coomassie Blue. Lane 1, 100 μg of PEG fraction from β-NF mitoplasts; lane 2, 20 μg of ω-octylamine agarose fraction; lane 3, 15–18 μg of DEAE column fraction; lane 4, 1.5 μg of protein from hydroxylapatite fractions 31–33 (see Fig. 3); lane 5 and 6, 0.5- and 3-μg proteins, respectively, from hydroxylapatite fractions 47–49 shown in Fig. 3; lanes 7–9, increasing concentrations (1, 2, and 3 μg, respectively) of proteins from fractions 31 to 33 as in lane 4; lanes 10 and 11, 1 and 3 μg of proteins from β-NF microsomal P-450 eluted from hydroxylapatite (fractions 55–59 in Fig. 3); and lane M, molecular weight markers of 55 kDa (glutamate dehydrogenase), 43 kDa (ovalbumin), 36 kDa (lactate dehydrogenase), and 29 kDa (carbonic anhydrase). B, spectra of CO-bound sodium dithionite-reduced mitochondrial P-450 shown in lanes 7–9, designated as P-450mt1 (--), and of P-450 shown in lanes 5 and 6, designated as P-450mt2 (±±±).

It is seen that both of these fractions contain a prominent protein band with migration pattern closely similar to that of P-450mt1 purified from β-NF-induced mitochondria (see Fig. 5, lane 6). It should also be noted that both of these fractions show a characteristic P-450 spectra at 450 nm (result not shown). Further, there is no detectable 54-kDa species characteristic of P-450mt2 in the control mitochondrial preparation. In repeated attempts we could not further purify the P-450 from control rat liver mitochondria due to very low yield and possible loss of heme during the purification steps. Assuming that the 52-kDa species is the P-450 hemoprotein, the fraction shown in Fig. 5 (lane 5) may be about 15% pure.

The purified mitochondrial cytochromes P-450mt1 and P-450mt2 were further compared and characterized using a polyclonal antibody to microsomal cytochrome P-450c which was shown to interact with a 54-kDa mitochondrial protein and inhibit mitochondrial aryl hydrocarbon hydroxylase activity (25) and a monoclonal antibody having exclusive specificity for microsomal P-450c (34). The Western blot analysis presented in Fig. 6A shows that the polyclonal antibody identifies a 54-kDa protein in the PEG fraction of β-NF-induced mitochondria (lane 2), but not in a similar fraction from control uninduced mitochondria (lane 1). The polyclonal antibody, however, fails to cross-react with purified P-450mt1 (lane 3) as well as with partially purified P-450 from control mitochondria (lane 6). However, it reacts with mitochondrial P-450mt2 and microsomal P-450c (lanes 4 and 5). In contrast, the monoclonal antibody to microsomal P-450c does not cross-react with either purified mitochondrial cytochromes P-450mt1 or P-450mt2 (see Fig. 6B, lanes 1 and 2) but specifi-
by SDS-PAGE and stained with Coomassie Blue as in Fig. 4. Column fraction; protein from the second half of the peak fraction from hydroxylapatite column (fractions 30 and 31); lane 5, 2 μg of protein from the second half of the peak fraction from hydroxylapatite (fractions 32 and 33); lane 6, 1 μg of protein from first half of peak fraction from hydroxylapatite column (fractions 32 and 33); and lane 7, 2 μg of P-450mt2 and lane M, molecular weight markers as described in Fig. 4, except that an additional marker of 95 kDa (phosphorylase b) was also run.

**FIG. 5.** Electrophoretic patterns of protein from control mitoplasts at various stages of purification. Proteins were analyzed by SDS-PAGE and stained with Coomassie Blue as in Fig. 4. Lane 1, 75 μg of PEG fraction from control uninduced mitoplasts; lane 2, 20 μg of α-octylamine agarose-bound protein; lane 3, 20 μg of DEAE column fraction; lane 4, 5 μg of protein from first half of peak fraction from hydroxylapatite column (fractions 30 and 31); lane 5, 2 μg of protein from the second half of the peak fraction from hydroxylapatite (fractions 32 and 33); lane 6, 1 μg of P-450mt1; lane 7, 2 μg of P-450mt2; and lane M, molecular weight markers as described in Fig. 4, except that an additional marker of 95 kDa (phosphorylase b) was also run.

**FIG. 6.** Western blot analysis of mitochondrial cytochromes P-450 using polyclonal and monoclonal antibodies to microsomal P-450c. PEG fractions and purified cytochromes P-450 from control mitoplasts and β-NF-induced mitoplasts as well as microsomes were probed with a polyclonal antibody to P-450c (28) and a monoclonal antibody (clone 1-7-1P, see Ref. 33) to P-450c. A: lane 1, 50 μg of protein from control mitochondrial PEG; lane 2, 50 μg of protein from β-NF mitoplast PEG fraction; lane 3, 3 μg of purified P-450mt1; lane 4, 3 μg of purified P-450mt2; lane 5, 3 μg of purified P-450c; and lane 6, 3 μg of partially purified P-450 from control mitochondria. B, lanes 1, 2, and 3 represent duplicates of lanes 3, 4, and 5, respectively, from A. Lanes in A were probed with polyclonal antibody, and lanes in B were probed with monoclonal antibody.

**FIG. 7.** Peptide fingerprint patterns of cytochromes P-450mt2 and P-450c. Purified enzymes (10 μg for each digestion) were digested with 1–2 μg of V8 protease from Staphylococcus aureus for various lengths of time, electrophoretically separated on a 16% polyacrylamide gel, transblotted to nitrocellulose membrane, and probed with polyclonal antibody to P-450c by the Western blot method as described under "Materials and Methods." Lanes 1, 2, and 6, P-450mt2 immediately after addition of protease; 10 and 20 min of digestion with protease, respectively; lanes 3–5, P-450c immediately after addition of protease, 10 and 20 min of digestion with protease, respectively. Arrows indicate variant peptide fragments.
to electrophoresis on a 15% polyacrylamide gel and transblotted onto nitrocellulose membrane as described under "Materials and Methods." The protein bands on the membrane were visualized by reversible staining with 0.1% Ponceau S in 1% acetic acid (A). The membrane was washed with repeated changes (3–4) of 20 mM Tris-HCl (pH 8.8) to remove the stain and subsequently probed with polyclonal antibody to P-450 from control mitochondria; lane 2, 10 pg of P-450 as in lane 1; lane 3, 2.5 μg of P-450mt1; and lane 4, 3 μg of P-450mt2. Molecular weight standards were run as described in Fig. 4.

+ ISPPr, and their activities were compared with microsomal P-450c reconstituted with NADPH-cytochrome P-450 reductase. As shown in Table II, cytochrome P-450c reconstituted with NADPH-P-450 reductase yields maximal aryl hydrocarbon hydroxylase activity of 1.8 nmol/nmol of P-450/min with DMBA as substrate and 4.6 nmol with benzo(a)pyrene as substrate. The control mitochondrial P-450 yields no significant benzo(a)pyrene metabolism in the presence of either NADPH-cytochrome P-450 reductase or Adx + Adr. When reconstituted with ISP + ISPPr, however, this P-450 yields marginal activity in the range of 0.2 nmol. Cytochromes P-450mt1 and P-450mt2 purified from β-NF-induced mitochondria show aryl hydrocarbon hydroxylase activity in the range of 0.22–0.42 when reconstituted with Adx + Adr, and nearly 2-fold higher activity (0.5–0.8 nmol) with ISP + ISPPr. Further, in confirmation of our previous observation (24), NADPH-cytochrome P-450 reductase is able to support the aryl hydrocarbon hydroxylase activities of both of these mitochondrial enzymes. In contrast, mitochondrial Adx + Adr or ISP + ISPPr are unable to support the activity of microsomal P-450c. It is also seen from Table II that control mitochondrial P-450 has an exclusive requirement for the Adx + Adr electron transfer system for DMBA activation as it shows negligible activity with NADPH-cytochrome P-450 reductase. Mitochondrial P-450mt2, on the other hand, shows DMBA metabolism (0.5–0.6 nmol) in the presence of both NADPH-cytochrome P-450 reductase or Adx + Adr. Furthermore, none of the mitochondrial enzymes shows activity for ethoxycoumarin de-ethylase while P-450c in the presence of NADPH-P-450 reductase yields 11.0-nmol activity.

Since hepatic mitochondria are known to contain activity for the metabolism of C-27 sterols, the ability of partially purified P-450 from control mitochondria and purified P-450mt1 and P-450mt2 to metabolize [3H]cholesterol and [3H]vitamin D3 was studied. The metabolic products formed under various incubation conditions were analyzed by HPLC, and the metabolites were identified by comparing with the retention profiles of known markers (see Figs. 9 and 10 in Miniprint). The HPLC patterns of [3H]cholesterol incubated in a complete reaction system without added P-450 and with added control mitochondrial P-450 and purified P-450mt1 are presented in Fig. 9, A–C, respectively. It is seen that both control mitochondrial P-450 and P-450mt1 metabolize [3H]cholesterol into 25-OH and 26-OH forms and an additional metabolite of unknown identity. Similarly, vitamin D3 incubated in a complete system without added P-450 (see b) and with added P-450mt1 (see c) were resolved on a straight phase column as shown in Fig. 10A (Miniprint). The patterns of vitamin D3 and 25-OH vitamin D3 standards on this system are shown in Fig. 10A (Miniprint). The 25-OH vitamin D3

![Comparison of P-450mt1 and control mitochondrial P-450 by Western blot analysis.](image)

**FIG. 8.** Comparison of P-450mt1 and control mitochondrial P-450 by Western blot analysis. Protein samples were subjected to electrophoresis on a 15% polyacrylamide gel and transblotted onto nitrocellulose membrane as described under "Materials and Methods." The protein bands on the membrane were visualized by reversible staining with 0.1% Ponceau S in 1% acetic acid (A). The membrane was washed with repeated changes (3–4) of 20 mM Tris-HCl (pH 8.8) to remove the stain and subsequently probed with polyclonal antibody to P-450 from control mitochondria; lane 2, 10 pg of P-450 as in lane 1; lane 3, 2.5 μg of P-450mt1; and lane 4, 3 μg of P-450mt2. Molecular weight standards were run as described in Fig. 4.

### TABLE II

**In vitro reconstitution of mitochondrial cytochromes P-450 with different electron transfer systems**

The reactions were carried out in 0.25-ml final volumes using 50 pmol of cytochrome P-450 and the electron transfer systems as stated under "Materials and Methods" for 15 min at 37 °C. The amounts of each of the substrates used per reaction vessel were: 25 nmol of [3H] benzo(a)pyrene (1 μCi/50 nmol), 25 nmol of [3H]DMBA (1 μCi/50 nmol), and 75 nmol of ethoxycoumarin. Other details were as described under "Materials and Methods."

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<td></td>
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<td>Control mitochondrial P-450</td>
<td>P-450mt1</td>
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*Values represent average of two separate estimates.

b NADPH P-450 reductase alone without added cytochrome P-450 gave undetectable activity with all these substrates.
and unmetabolized vitamin D₃ eluted from the straight phase column were further resolved on a reverse phase C-18 column (Fig. 10B, Miniprint). It is seen from Fig. 10, A and B, that the 25-OH vitamin D₃ peak collected from the first column eluted as a single peak on the second column. These results are in complete agreement with a recent observation by Reddy et al. (47). For this reason, the radioactivity profile of straight phase HPLC (disintegrations/min in 25-OH vitamin D₃/total disintegrations/min) was used for calculating the enzyme activity. The results of these assays summarized in Table III shows that ISP + ISP₃ without added P-450 yield negligible activity for vitamin D₃ and cholesterol metabolism. It is also seen that P-450 from control mitochondria as well as P-450mt1 and P-450mt2 show nearly comparable activities for 25-plus 26-hydroxylation of cholesterol in the range of 1.1–1.3 nmol. Furthermore, P-450mt1 and P-450mt2 show 2–2.5-fold (40 and 51 pmol/nmol of P-450/min, respectively) activity of the control mitochondrial P-450 (18 pmol/nmol of P-450/min) for 25-hydroxylation of vitamin D₃. In contrast, the microsomal P-450 shows no significant activity with both of these substrates. These results show that the purified mitochondrial P-450 isoforms can metabolize both the xenobiotic agents like benzo(a)pyrene and DMBA and also the C-27 steroids like cholesterol and vitamin D₃, and in this respect show some contrasting difference from microsomal P-450c.

**DISCUSSION**

Mitochondrial P-450 from bovine adrenal cortex, corpus luteum, and kidney have been purified and characterized to various degrees with respect to substrate specificity and electron transfer systems (12–16). Despite observations that hepatic mitochondria contain P-450-type monoxygenase activity to metabolize C-27 steroids (18–20) as well as varied xenobiotic compounds (22–24), a cytochrome P-450 enzyme has not yet been successfully purified to homogeneity from these organelles. A major problem in this direction appears to be the low P-450 content of hepatic mitochondria (0.05–0.07 nmol of P-450/mg of protein) and extreme sensitivity of the enzyme resisting purification. Previous results from our laboratory (24) as well as from others (20, 21) showed the possible induction of hepatic mitochondrial P-450 by known inducers like 3-MC and phenobarbital (20, 24). In this paper, we report on the purification of two distinct forms of mitochondrial P-450 from β-NF-induced rat liver, with apparent molecular masses of 52 kDa (P-450mt1) and 54 kDa (P-450mt2).

The purified P-450mt2 comigrates with the major β-NF-induced microsomal P-450 (P-450c) on SDS-PAGE and cross-reacts with polyclonal antibody to P-450c. Although not presented, the polyclonal antibody also inhibited the activity of P-450mt2 to metabolize various substrates in an in vitro reconstituted system. For these reasons, the molecular properties of P-450mt2 were compared with those of P-450c by Western blot analysis and peptide fingerprint analysis. As shown in Fig. 6B, a monoclonal antibody, highly specific for microsomal P-450c, fails to interact with P-450mt2 suggesting significant structural differences. Further, as shown in Fig. 7, the patterns of polyclonal antibody reactive peptides generated by limited digestion of the two isoforms with V8 protease show distinct differences providing further support to the possibility that the two enzymes in question represent distinct molecular species. With a view to obtain direct evidence for this possibility, we have attempted to compare the N-terminal sequences of the two P-450 isoforms. The microsequencing by Edman degradation of phenylhydantoin-derivated P-450c has yielded sequences (10 cycles determined as: Pro-Ser-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Thr) identical to those previously reported for this isoform (48, 49). However, in repeated attempts the N terminus of P-450mt2 was not amenable to sequence analysis by phenylthiodyantoin derivatization suggesting that the N terminus is blocked. Attempts are currently under way to determine the nature of this blockage. Another line of evidence on the DEAE elution profile (Fig. 2) showing possible charge differences between P-450c and P-450mt2 also supports the view that these represent two different isoforms. Cytochrome P-450mt2 is eluted at lower salt concentration (60–80 mM NaCl as opposed to 100–150 mM for P-450c), suggesting that it carries a lesser number of negatively charged groups. This observation is in keeping with our recent results (25) showing that the anti-P-450c IgG-reactive 54-kDa P-450 in 3-MC-induced rat hepatic mitochondria has an apparent pl of 6.3 as compared to P-450c which has a pl of 5.9 (25).

The catalytic properties of the two P-450s purified from β-NF-induced mitochondria were compared with the activity of the partially purified P-450 from control mitochondria and also microsomal P-450c. As shown in Table II, the activation of benzo(a)pyrene by P-450mt1 and P-450mt2 was less than (about one-tenth) that of P-450c. Further, cytochrome P-450c (Table II) and other microsomal isoforms show varied levels of ethoxychoumarin de-ethylase activity (31). In contrast, however, this activity was not detectable in any of the mitochon-

### Table III

**Metabolism of vitamin D₃ and cholesterol by purified cytochromes P-450**

<table>
<thead>
<tr>
<th>Description</th>
<th>25-OH + 26-OH cholesterol formed</th>
<th>25-OH vitamin D₃ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>nmol·nmol⁻¹·P₄50⁻¹·min⁻¹</em></td>
<td><em>pmol·nmol⁻¹·P₄50⁻¹·min⁻¹</em></td>
<td></td>
</tr>
<tr>
<td>ISP + ISP₃ alone</td>
<td>0.37</td>
<td>0.0</td>
</tr>
<tr>
<td>Control mitochondrial P-450 + ISP + ISP₃</td>
<td>1.09</td>
<td>18.8</td>
</tr>
<tr>
<td>P-450mt1 + ISP + ISP₃</td>
<td>1.2</td>
<td>51.3</td>
</tr>
<tr>
<td>P-450mt2 + ISP + ISP₃</td>
<td>1.31</td>
<td>40.2</td>
</tr>
<tr>
<td>P450c + NADPH P-450</td>
<td>0.04</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Values represent mean of two independent estimates.

NADPH P-450r, NADPH-cytochrome P450 reductase.
drial forms under study indicating a distinct difference in substrate specificity. Another major difference between the mitochondrial cytochromes P-450 and microsomal P-450c is in their ability to hydroxylate C-27 sterols. As shown in Table III, all three mitochondrial enzymes show nearly comparable activity for C-25 and C-26 hydroxylation of cholesteryl. A similar activity and metabolite pattern was obtained by Pedersen et al. (20) using a system reconstituted with PEG-fractionated mitochondrial extracts. All three mitochondrial enzymes, however, show varying levels of activity for 25-

hydroxylation of vitamin D₃ (see Table III). The control mitochondrial P-450 shows a relatively low activity (18 pmol/ nmol of P-450/min) while P-450mt1 and P-450mt2 show 2-

2.5-fold higher activities. These results are consistent with reports showing a low activity and high saturation nature of hepatic mitochondrial activity for 25-hydroxylation of cholecalciferol (for reviews see Refs. 19 and 21). Aslo, this activity pattern is quite different from the partially purified P-450 from control mitochondria. In addition, its oxidation of benzo(a)pyrene and 25-hydroxylation of vitamin D₃ than the P-450 from control mitochondria. In addition, its oxidized pattern is quite different from a male-specific P-450 purified from control mitochondria, on the other hand, showed significantly higher catalytic activity for the aryl hydrocarbon hydroxylase activity of the partially purified P-450 from control mitochondria, on the other hand, requires Adx + Adr or ISP + ISPr for activity, and it is minimally active with NADPH-cytochrome P-450 reductase (Table II). In this respect, our results are in agreement with those previously reported by Sato et al. (51). 3) The unique property of P-450mt1 and P-450mt2 purified from β-NF-

-induced mitochondria is that they are active with both Adx + Adr and NADPH-cytochrome P-450 reductase, although they show significant preference for liver mitochondrial ISP + ISPr. The NADPH-cytochrome P-450 reductase used in this study consists of a nearly homogeneous 75-76-kDa protein on SDS-PAGE with no detectable lower molecular weight contaminants. Thus, contaminating ISP and ISPr may not be the reason for the observed reconstitution of the two mitochondrial isoforms with the NADPH-cytochrome P-450 reductase system. These results confirm our previous observation that the aryl hydrocarbon hydrolase activity of the PEG fraction from 3-MC-induced mitochondria can be supported by both mitochondrial type (Adx + Adr) and microsomal-type (NADPH-cytochrome P-450 reductase) electron transfer systems (24).

Based on the initial results showing the presence of a protein in partially purified P-450 from control mitochondria which exhibits an SDS-PAGE pattern similar to purified P-450mt1 (Fig. 5), it was felt that the latter P-450 is a constitutive form induced and stabilized by β-NF. P-450mt1, however, showed significantly higher catalytic activity for the oxidation of benzo(a)pyrene and 25-hydroxylation of vitamin D₃ than the P-450 from control mitochondria. In addition, its distinct activity to accept electrons through NADPH-cytochrome P-450 reductase suggests that P-450mt1 may be different from the partially purified P-450 from control mitochondria. The Western blot experiments (Fig. 8) showing negligible cross-reactivity of a polyclonal antibody to P-450mt1 with control mitochondrial P-450 support this latter possibility. Further, experiments presented in Fig. 6 show that P-450mt2 is a truly induced form not present in significant levels in control mitochondria. It is becoming increasingly apparent that a number of hepatic microsomal constitutive forms of P-450 as well as the inducible ones share varied levels of homologies at the level of DNA and protein sequences (52-55). Recent studies of Nagata et al. (56) show that some members of the P-450a family are evolved by exon shuffling and gene conversion. Available evidence with the adrenal cortical P-450 isoforms, however, suggest minimal conservation of sequence between the mitochondrial and microsomal forms (57). In view of this, extensive immunochemo-hology between P-450mt2 and P-450c presents an unusual evolutionary trend. In summary, results reported in this paper provide direct evidence for the induction of two distinct P-

450 isoforms in rat hepatic mitochondria.

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REFERENCES


355
Cytochrome P-450 from Hepatic Mitochondria

9541

stopped by the addition of 30 μL of 10% perchloric acid containing 5% trichloroacetic acid and then keeping in ice-water for 3 min. The protein fragments were subjected to SDS-PAGE on a 3% gel and selectively detected by Western blotting using polyclonal antibody to P-450 as described above.

Reconstitution of P-450 Activity: The activities of the mitochondrial enzymes to metabolize different substrates were reconstituted essentially as described before (26). The reaction mixture contained 0.08 M potassium phosphate buffer (pH 7.4), 3.5 μM MgCl2, 0.5 mM EDTA, 2 mM NaF, 17 mM glucose, 85 μM [14C] nicotinamide adenine dinucleotide (4.4 μmol protein), 45 or 50 pmol purified P-450 and 10 μg dilauroylphosphatidylcholine in a final volume of 250 μL. NADH-cytochrome P-450 reductase was added. In some experiments 0.5 mM and 15 mM P-450 were added to reconstitute the P-450 activity. The reaction was initiated by adding the various substrates at concentrations specified in Table I and II, and carried for 1 to 20 min at 37°C or 37°C or 5°C in a sealed water bath. In the case of [1]NADPH and [2]NADH the reaction was stopped by adding two volumes of 0.1 M HCl in 0.5% 2,6-dichlorophenolindophenol and the 2,6-dichlorophenolindophenol activity was measured by the phase partition method of Van Geertruyden et al. (39). In the case of ethoxycoumarin as substrate, the activity was added with 0.2 and 0.1 mg of ethoxycoumarin and deoxyribonuclease activity was measured in a fluorimeter according to Groux and Pololet (40). In assays using [1]vitamin D3 and [2]cholesterol, the reaction was stopped by adding 4.5 μL of methanol/ chloroform (6:3.2 v/v) to yield a single phase (20). The metabolites were extracted and quantitated by the HPLC analysis described below.

High Pressure Liquid Chromatography Analysis: The metabolic products of vitamins D3 were analyzed by the two step HPLC involving a straight phase separation on a 5.8 x 25 cm Zorbax-Sil column (DuPont Instruments, Wilmington, DE) followed by a reverse phase analysis on a Bondapak C18 column (Waters Associates, Milford, MA). The straight phase HPLC was developed with a solvent system of 75% propylene in hexane and the reverse phase was developed with an isocratic solvent system of 85% methanol (41). We have used [1]H-labeled substrates in our assays to circumvent the complication of non-specific 37 absorbance components which are normally present in the organic phase. The procedure for extraction of vitamin D metabolites and chromatography on straight phase and reverse phase columns essentially as described by Burger-Teindl et al. (41). The cholesterol metabolites were extracted from the [2]H-incubations as described by Klugh and Spyer (42) and resolved on a Zorbax-Sil column by the procedure described by Pedersen et al. (43). The elution times of each metabolite was determined by absorbance at 254 nm and by radioactivity measurements using a continuous flow liquid scintillation system, Handsil 5000 (Hawksil Corporation). The [1]H cholesterol and [2]H vitamins D3 metabolites in the reaction mixtures were quantitated by comparing the radioactivity in the metabolite peaks (peak areas) with the total radioactivity detected in the chromatogram. The radioactivity recovered in the metabolites peaks were corrected for the rates of recovery of internal standards (i.e., absorbance and radioactivity) which was about 100 in repeated runs.

Other Methods: Cholesterol P-450 content was measured according to Osuna and Sato (44) using extinction coefficient of 91 cm⁻¹·mol⁻¹·L⁻¹ for sodium diclohexate reduced CO bound P-450. NADP-dependent reductase activity was assayed by the method of Higgins and Storen (45). Protein was measured according to the method of Lowry et al. (46).

Figure 1: Western blot analysis of mitochondrial proteins from induced rat liver, liver mitochondria were isolated from rats pretreated with 5-HC (24) or NBP, extracted with dichloromethane and fractionated with urea as described under "Materials and Methods". Mitochondrial proteins were analyzed by SDS-PAGE and transferred to nitrocellulose membranes which were stained with Coomassie Brilliant Blue or visualized by Western blotting using polyclonal antibody to P-450. The blot was exposed to X-ray film for 24 h at −70°C. The molecular weights were determined by the method of Lowry et al. (46). Figure 2: DEAE-Sephadex chromatography of mitochondrial and microsomal cytochrome P-450. NADPH-dependent oxygen bound fractions from control rat liver mitochondria as well as 5-HC-induced mitochondrial fractions were chromatographed as shown in Table I. NADPH-dependent oxygen bound fractions from 5-HC-induced mitochondrial fractions were chromatographed as described under "Materials and Methods". The fractions of 5-HC-induced mitochondrial cytochrome P-450 were separated by DEAE-Sephadex chromatography as described previously (25). SDS-PAGE (2μg each) or immunoprecipitated proteins (0.2μg each) were plated on a polyacrylamide gel of 5% and applied to the column in 50-100 μL of 2% propylene in hexane as described by Burger-Teindl et al. (41). The fractions were collected on a Zorbax-Sil column using 75% propylene in hexane. 15% of the column was eluted with 75% propylene in hexane and the remaining 85% was eluted with 85% methanol (41). The elution times were determined by the method of Higgins and Storen (45). The fractions were collected by a fraction collector. Fractions corresponding to vitamin D3 and 25-OH D3 peaks in the first column (A) were eluted under 25°C and used for the reverse phase Bondapak-C18 column (B). The reverse phase column was developed with an isocratic solvent system of 85% methanol and 0.2% of the eluate was visualized by ninhydrin for visualization detection. The retention times for vitamin D3 and 25-OH vitamin D3 standards are indicated with arrows.

Figure 3: Hydroxylation chromatography of mitochondrial and microsomal cytochrome P-450. The peak fractions from DEAE column shown in Figure 2 were purified by preparative HPLC (20). The column was developed with a step gradient of potassium phosphate buffer at 40, 60, 90 and 180 mM concentrations and fractions of 20 μL each were collected and monitored for absorbance at 280 nm. Open circles (O) represent the elution pattern of NBP-induced microsomal cytochrome P-450 and closed circles (●) represent the pattern of 5-HC-induced mitochondrial cytochrome P-450.