Hepatic Mitochondrial Cytochrome P-450 System

IDENTIFICATION AND CHARACTERIZATION OF A PRECURSOR FORM OF MITOCHONDRIAL CYTOCHROME P-450 INDUCED BY 3-METHYLCHOLAN-THRENE

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Hepatic mitoplasts from 3-methylcholanthrene-treated rats contain cytochrome P-450 which can metabolize polycyclic aromatic hydrocarbons like benzo(a)pyrene. Mitochondrial cytochrome P-450 was partially purified and reconstituted in vitro using adrenodoxin and the adrenodoxin reductase electron transfer system and [3H]benzo(a)pyrene as the substrate. A polyclonal antibody to purified microsomal P-450c (a major 3-methylcholanthrene-inducible form) inhibited the activity of mitochondrial enzyme in a concentration-dependent manner and also reacted with a 54-kDa protein on the immunoblots. A monoclonal antibody having exclusive specificity for P-450c, on the other hand, did not inhibit the aryl hydrocarbon hydroxylase activity of the mitochondrial enzyme and showed no detectable cross-reaction with the 54-kDa mitochondrial protein. Similarly, two-dimensional analysis and immunodetection using the polyclonal antibody showed distinct molecular properties of the mitochondrial enzyme different from the similarly induced microsomal P-450c with respect to the isoelectric pH. In vitro translation of free polysomes from 3-methylcholanthrene-induced liver, transport of precursor proteins by isolated mitochondria in vitro, and immunoprecipitation with the polyclonal antibody showed the presence of a 57-kDa putative precursor which is transported and processed into mature 54-kDa species. These results present evidence for the true intramitochondrial location of the P-450c-antibody reactive isofrom detected in 3-methylcholanthrene-induced rat liver mitochondria.

Mitochondria from different steroidalogenic tissues (1-4), kidney (5-7), and liver (8-14) contain cytochrome P-450 enzymes which are involved in the oxidation of physiologically important compounds such as cholesterol, vitamin D3, and bile acids. These mitochondrial cytochromes P-450 have specific requirements for mitochondrial type ferrodox (an iron-sulfur protein) and ferrodoxin reductase (a flavoprotein) for activity (10, 12, 15-17), and in most cases, they are inactive with microsomal NADPH cytochrome P-450 reductase (17). Recent studies in our laboratory showed that mitochondrial cytochrome P-450 components are induced by known inducers of microsomal cytochrome P-450, such as phenobarbital and 3-methylcholanthrene (18, 19). The cytochrome P-450 solubilized from the mitoplast preparations from induced rat liver was able to activate carcinogens like aflatoxin B1 and benzo(a)pyrene in an in vitro reconstituted system (19). The mitochondrial association of these inducible cytochrome P-450s was deduced from the in vitro reconstitution of activity with the mitochondria-specific adrenodoxin and adrenodoxin reductase electron carrier system and also by the purity of mitochondrial preparations (19). The adrenodoxin- and adrenodoxin reductase-supported hydroxylation of benzo(a)-pyrene and aflatoxin B1 was significantly inhibited by antibodies to purified microsomal cytochrome P-450c (a major 3-methylcholanthrene-inducible form) and cytochrome P-450b (a major phenobarbital-inducible form), respectively, suggesting partial immunohomoology between the mitochondrial and microsomal counterparts (19). In this study, we have further characterized the 3-methylcholanthrene-induced mitochondrial enzyme using polyclonal and monoclonal antibodies to similarly induced microsomal cytochrome P-450c.

EXPERIMENTAL PROCEDURES

Materials—The biochemicals and enzymes used were of highest purity grades available from Fluka Chemical Corp., Fisher, or Sigma. Reagents for polycrystallide gel electrophoresis were purchased from Bio-Rad. Nitrocellulose membrane for blot transfer was from Schleicher & Schuell. CNBr-activated Sepharose 4B was from Pharmacia LKB Biotechnology Inc. IgGorb (protein A-coated cells) for immunoprecipitation was from The Enzyme Center Inc. Immuno blotting system including alkaline phosphatase-conjugated anti-rabbit goat IgG, anti-mouse rabbit IgG, and the reagents for color development were purchased as a kit from Promega Biotech Corp. Ampholine mixture for the isoelectric focusing was purchased from Pharmacia LKB Biotechnology Inc. [3H]Benzo(a)pyrene (40 Ci/mmoll), [36S]methionine (>1000 Ci/mmoll), and [125I]-labeled protein A were purchased from Amersham Radiochemical Corp.

Isolation of Cytochrome P-450 Fractions—Male Sprague-Dawley rats weighing about 150 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used in all of the experiments presented in this paper. A group of 10-12 rats was administered intraperitoneally with 3-methylcholanthrene in corn oil for 3 days (30 mg/kg/day). Control rats received equivalent volumes of corn oil. About 16-20 h after the third treatment the rats were killed by decapitation, and the livers were...
removed and washed free of blood clots with ice-cold 0.9% NaCl solution and used for the isolation of mitochondria and microsomes (20). Mitochondria were isolated using the sucrose-mannitol buffer, and the outer membrane was removed by digitonin treatment (21). The resultant mitochondrial preparations containing less than 0.6% of microsomal-specific (rotenone-insensitive) NADPH cytochrome c reductase activity were separated by gradient centrifugation through a 30% of glucose-6-phosphate buffer solution at 100,000 g for 1 h at 4 °C. The microsomal cytochrome P-450 was solubilized and fractionated with PEG by using a procedure similar to the one described for the mitochondrial enzyme. Purification of over 90% homogeneous cytochrome P-450c from 3-methylcholanthrene-induced rat liver microsomes and preparation of monospecific antibody (polyclonal) in rabbits was as described by Wilson et al. (22). The development and characterization of monoclonal antibody to cytochrome P-450c were described by Park et al. (23).

In Vitro Reconstitution of Cytochrome P-450 Activity—Mitochondrial and microsomal enzymes were reconstituted in vitro in final volumes of 0.25 ml using bovine adrenodoxin and adrenodoxin reductase and NADPH cytochrome P-450 reductase, respectively. NADPH was generated in a reaction mixture containing 0.08 M potassium phosphate buffer (pH 7.4), 3.3 mM MgCl₂, 0.2 mM EDTA, 2 mM NADP, 17 mM isocitrate, and 80 μg/mi isocitrate dehydrogenase (4.4 units/mg of protein) at 37 °C for 10 min. Mitochondrial or microsomal cytochrome P-450 (10–15 pmol), adrenodoxin (0.4 nmol), adrenodoxin reductase (0.045 nmol), or NADPH cytochrome P-450 reductase (0.2 nmol/mg), and 12–10 nmol of [3H]benzo(a)pyrene (20 mCi/mmol) as the substrate were added, and the incubation was continued for 30 min. The reaction was stopped by adding 2 volumes of 0.1 N KOH in 85% dimethyl sulfoxide, and the [3H]benzo(a)pyrene metabolites were analyzed using the phase partition method of Van Cantfort et al. (24).

In experiments involving the immunotitration of cytochrome P-450c, the enzyme preparations were preincubated with the appropriate antibody for 15 min at 25 °C before using in the reconstituted metabolic assays (19). The characterization and specificities of the two antibodies used in this study, namely the polyclonal antibody to purified cytochrome P-450c and monoclonal antibody to cytochrome P-450c, have been described previously by Wilson et al. (22) and Park et al. (23), respectively. The monoclonal antibody (1-7P) was a generous gift from Drs. S. S. Park and Harry Gelboin, National Institutes of Health, Bethesda, MD.

Immunoaffinity Purification of Cytochrome P-450—Polyclonal antibody covalently linked to CNBr-activated Sepharose 4B was used to purify cytochrome P-450c from microsomal and mitochondrial PEG fractions essentially as described by Park et al. (25). About 5 mg of IgG purified by adsorption to protein A-Sepharose according to the manufacturer’s suggested procedure was covalently bound to 0.5 g of CNBr-activated Sepharose 4B as described previously (26). The Sepharose-bound antibody was packed in a 0.5 × 7.0-cm column and equilibrated with 20 mM sodium phosphate buffer (pH 7.5) containing 20% glycerol, 0.5% sodium cholate, and 0.1% Triton N-101 (buffer A). About 150 mg of mitochondrial or microsomal PEG fraction was dissolved in 15 ml of buffer A containing 0.5 mM EDTA, centrifuged at 100,000 × g, and the insolubles, and passed through the antibody column at the rate of 1 ml/min. The column was recycled through the column two more times. The column was washed extensively with buffer A until no protein was detected in the eluate. The bound protein fraction was eluted with 5 ml of 0.1 M glycine buffer (pH 2.8) containing 0.15 M NaCl and 0.3% sodium cholate, dialyzed against 20 mM sodium phosphate buffer (pH 7.5 containing 1 mM dithiothreitol), concentrated to <0.3 ml by filtration through the Amicon membrane filtration system, and adjusted to 20% with glycerol and stored at −70 °C.

Immunoblot Analysis of Proteins—Protein samples were subjected to two-dimensional gel electrophoresis using the procedure of O’Farrell (29). Briefly, proteins were separated under reducing conditions in the first dimension on 4% polyacrylamide gels containing 8 M urea and 2% ampholytes (pH 5–8) by isoelectric focusing, and resolved by electrophoresis on 12% polyacrylamide slabs in the presence of SDS and transferred to nitrocellulose membranes. The membranes were probed with the appropriate primary antibody and alkaline phosphatase-conjugated secondary antibody. Immunoreactive proteins were identified using chromogenic substrates as described in the manufacturer’s protocol (Promega Biotech Inc.). In some experiments, the immunoblots were developed using 5 μCi (>30 mCi/mg) of [3H]labeled protein A (30), and the radioactive bands were detected by exposure to x-ray films.

In Vitro Transport of Proteins into Mitochondria—Free polyelectrolyte from control and 3-methylcholanthrene-induced rat livers were isolated according to the method of Raymond and Shore (31), suspended in a buffer containing 20 mM Hepes (pH 7.6), 150 mM KCl, 7 mM mercaptoethanol, 5 mM Mg(CH₃COO), at a final concentration of 10 mg/ml as described before (33). Polysomes were translated in the micrococcal nuclease-treated rabbit reticulocyte system (32) in the presence of 1 μCi [35S]methionine (>1000 Ci/mmol). After 60 min of incubation at 28 °C, protein synthesis was terminated by adding 150 μg/ml cycloheximide and 3 mg/ml unlabeled l-methionine, and the incubations was continued for 15 min to release the peptide chains. The in vitro transport of translation products was carried out essentially as described before using mitochondria washed 3 times with the isolation buffer (33). A typical transport reaction involved the mixing of 30 μl of reticulocyte lysate containing the translation products (2–4 × 10⁶ cpm) with 60 μl of the modified sucrose-mannitol buffer containing 180–200 μg of freshly isolated mitochondria with intact outer membrane. The reaction mixture was incubated at 27 °C for 60 min and treated with 200 μg/ml each of trypsin and chymotrypsin as described before (33). Inactivation of proteases by treatment with inhibitors and separation of mitochondrial by pelleting through 1.2 M sucrose were essentially as described earlier (33). The resultant mitochondrial pellet was washed twice with mitochondrial isolation buffer and used for immunoprecipitation.

Immunoprecipitation—The reticulocyte lysate samples were processed for immunoprecipitation as described by Bhat and Avedbani (33). Mitochondrial samples were dissociated by boiling in a buffer containing 1% SDS and subsequently diluted 10-fold to reduce the effective SDS concentration to 0.2% as described by Matacha and Waterman (34). The rest of the procedures for immunoprecipitation and recovery of immunoprecipitates were as described earlier (33). The immunoprecipitates were subjected to electrophoresis on 12% polyacrylamide-SDS gels, and the labeled proteins were detected by fluorography using EN’HANCE (Du Pont-New England Nuclear) and subsequent exposure to Kodak SB5 x-ray films.

RESULTS

Effects of Polyclonal and Monoclonal Antibodies on the Activity of Mitochondrial Cytochrome P-450—Previous studies from our laboratory showed that polyclonal antibody to microsomal P-450c inhibited both the NADPH cytochrome P-450 reductase-supported and adrenodoxin/adrenodoxin reductase-supported enzyme activities of the similarly induced mitochondrial cytochrome P-450 (19). However, immunocytochemical studies showed no significant cross-reactivity of monoclonal antibody raised against cytochrome P450c with mitochondrial inner membrane. Therefore, these two antibodies further verify the similarities or differences between the mitochondrial and microsomal isoforms induced by 3-methylcholanthrene.

As shown in Table I, the 3-methylcholanthrene-induced mitochondrial enzyme can metabolize significant levels of [3H]benzo(a)pyrene (0.5 nmol/mmol of cytochrome P-450/ min) in an in vitro system reconstituted with bovine adreno- doxin/adrenodoxin reductase. Although not shown, omission of adrenodoxin and adrenodoxin reductase from the reaction mixture yields only a marginal activity in the range of 0.03–0.06 nmol/mmol of cytochrome P-450/min. Polyclonal anti-

1 The abbreviations used are: PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

2 H. V. Gelboin, personal communication.
body to microsomal cytochrome P-450c inhibits the activity of both mitochondrial and microsomal isoforms by 32–71% in a concentration-dependent manner (see Table I). Similarly, in agreement with the observations of Park et al. (23), the monoclonal antibody inhibits the activity of microsomal cytochrome P-450c (see Table I). However, the same antibody has no significant inhibitory effect on the adrenodoxin/adrenodoxin reductase-supported activity of the 3-methylcholanthrene-induced mitochondrial cytochrome P-450. Although not shown here, this antibody also has no inhibitory effect on the NADPH cytochrome P-450 reductase-supported activity of the mitochondrial cytochrome P-450. These results showing the differential effects of polyclonal and monoclonal antibodies suggest that similarly induced mitochondrial and microsomal cytochrome P-450 may have significant structural differences.

Characterization of Mitochondrial P-450 Using Antibody Probes—The molecular properties of 3-methylcholanthrene-induced mitochondrial cytochrome P-450 were compared with the microsomal P-450c by immunoaffinity purification and Western blot analysis. As shown in Fig. 1, the PEG-fractionated mitochondrial and microsomal proteins as well as the proteins bound to Sepharose-linked polyclonal antibody to cytochrome P-450c were compared by electrophoretic analysis on a polyacrylamide-SDS gel. Lanes 4 and 5 represent the patterns of PEG fractions from 3-methylcholanthrene-induced microsomes and mitochondria, respectively, which were used as the starting materials for the affinity purification. Microsomal protein bound to the antibody column resolves as a major band with an average molecular mass of 54 kDa (lane 1). The cholate-solubilized mitochondrial protein bound to the antibody column migrates with an apparent molecular weight nearly indetical to that of microsomal cytochrome P-450c (lane 2). Further, both of these proteins eluted from the antibody column comigrate with cytochrome P-450c purified by the conventional chromatography procedure (lane 3). Due to poor yield and possible denaturation during the purification we were unable to reconstitute the activity of immunoaffinity-purified mitochondrial enzyme. The nearly identical electrophoretic migration of the two cytochrome P-450 isoforms prompted us to compare the nature of these two proteins by Western blot analysis. As shown in Fig. 2, proteins from

### Table I

<table>
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<tr>
<th>Additions</th>
<th>AHH activity (nmol BaP metabolized/nmol P-450/min)</th>
<th>Mitochondria</th>
<th>Microsome</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>0.5</td>
<td>7.5</td>
<td></td>
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<tr>
<td>Preimmune sera, 0.2 mg</td>
<td>0.47 (6%)</td>
<td>6.9 (8%)</td>
<td></td>
</tr>
<tr>
<td>Polyclonal antibody</td>
<td>0.34 (32%)</td>
<td>4.8 (36%)</td>
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<td>0.10 mg</td>
<td>0.15 (70%)</td>
<td>2.2 (71%)</td>
<td></td>
</tr>
<tr>
<td>0.20 mg</td>
<td>0.49 (2%)</td>
<td>5.3 (29%)</td>
<td></td>
</tr>
<tr>
<td>Monoclonal antibody</td>
<td>0.47 (6%)</td>
<td>4.1 (45%)</td>
<td></td>
</tr>
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</table>

**FIG. 1.** Electrophoretic analysis of mitochondrial and microsomal cytochrome P-450 purified by immunoaffinity adsorption. Proteins were purified by affinity adsorption to Sepharose-linked anti-cytochrome P-450c antibody, and P-450c was purified by the conventional column chromatography method as described under “Experimental Procedures.” Protein samples were subjected to electrophoresis on 12% polyacrylamide-SDS gels and stained with Coo massie Brilliant Blue. One to two μg of affinity-purified mitochondrial P-450 (lane 1), microsomal cytochrome P-450 (lane 2), and purified cytochrome P-450c (lanes 3) and 60 μg of microsomal (lane 4) and 100 μg of mitochondrial (lane 5) PEG fractions were used for electrophoresis.

**FIG. 2.** Western blot analysis of proteins. Mitochondrial and microsomal PEG fractions from 3-methylcholanthrene-induced rat liver and immunoaffinity-purified cytochrome P-450 were analyzed by the Western blot using polyclonal and monoclonal antibodies to cytochrome P-450c. A, 25 μg each of the PEG fractions from control and 3-methylcholanthrene-induced livers were used for the analysis, and the blot was probed with polyclonal antibody and developed with 5 μCi of 125I-labeled protein A as described under “Experimental Procedures.” The polyclonal antibody-reactive bands were detected by exposing the membrane to x-ray films for 4–6 h. Lane 1, control mitochondria; lane 2, control microsome; lane 3, 3-methylcholanthrene-induced mitochondria; and lane 4, 3-methylcholanthrene-induced microsome. B, 2 μg each of affinity-purified cytochrome P-450 from 3-methylcholanthrene-induced livers were analyzed by the Western blot method, and the blot was developed with the Protoblot system (a kit from Promega Biotec). Lanes 1 and 3, microsomal cytochrome P-450; and lanes 2 and 4, mitochondrial cytochrome P-450. The blot in lanes 1 and 2 was probed with polyclonal antibody and the blot in lanes 3 and 4 with the monoclonal antibody. In all cases the antibody was diluted 1:1000.
Distinctive Properties of Hepatic Mitochondrial Cytochrome P-450

microsomal and mitochondrial PEG fractions, as well as the immunoaffinity-purified fractions, were resolved on 12% polyacrylamide-SDS gels, electrophoretically transblotted onto nitrocellulose membrane, and probed with polyclonal and monoclonal antibodies. As shown in Fig. 2A no significant polyclonal antibody-reactive protein is detected in the control mitochondrial PEG fraction (lane 1) although two minor protein bands are detected in the control microsomal fraction (lane 2). In agreement with the immunoaffinity purification results presented in Fig. 1, the polyclonal antibody identifies 54-kDa protein bands in both mitochondrial and microsomal PEG fractions from 3-methylcholanthrene-induced rat liver (Fig. 2A, lanes 3 and 4). Similarly, as shown in Fig. 2B, the polyclonal antibody identifies the immunoaffinity-purified microsomal as well as mitochondrial proteins (see lanes 1 and 2) having an identical electrophoretic migration pattern. Furthermore, in support of the results of enzyme inhibition presented in Table I, the monoclonal antibody interacts well with the affinity-purified microsomal cytochrome P-450c (Fig. 2B, lane 3) as well as the 3-methylcholanthrene-induced microsomal PEG fraction (results not presented). However, the same antibody fails to cross-react with the similarly induced mitochondrial PEG fraction (results not presented) or affinity-purified mitochondrial protein (see lane 4). These results support the possibility that although the 3-methylcholanthrene-induced mitochondrial cytochrome P-450 and microsomal cytochrome P-450c have nearly identical molecular weights and share some immunochemical characteristics, they have inherent structural differences as revealed in the epitope variations.

Comparison by Isoelectric Focusing—The possible differences between the 3-methylcholanthrene-induced mitochondrial and microsomal cytochrome P-450 were verified by immunodetection of the isoforms separated on the O'Farrell's type of two-dimensional gels involving isoelectric focusing in the first dimension and electrophoretic resolution on polyacrylamide-SDS gel in the second dimension. Proteins thus separated were electrophoretically transferred onto nitrocellulose membrane and probed with polyclonal antibody. As shown in Fig. 3A, the 3-methylcholanthrene-induced microsomal cytochrome P-450c resolves as a major component with an apparent pI of 5.7-6.0 and molecular mass of 54 kDa, whereas the similarly induced mitochondrial isoform resolves as a single component with an apparent pI of 6.2-6.5 and molecular mass of 54 kDa (see Fig. 3B). Furthermore, the mitochondrial and microsomal cytochromes P-450 resolve into distinct components when applied as a mixture (see Fig. 3C). These results indeed show that the mitochondrial and microsomal cytochrome P-450s induced by 3-methylcholanthrene are two independent proteins having distinct immunochemical characteristics and isoelectric points.

Identification of Precursor Forms and in Vitro Transport into Mitochondria—Proof for the intramitochondrial location of the minor species of P-450 associated with 3-methylcholanthrene-induced mitochondria was sought using an in vitro mitochondrial transport system. 35S-Labeled in vitro translation products programmed with cytoplasmic free polyribosomes from 3-methylcholanthrene-induced rat liver containing the putative precursors of mitochondrial proteins were incubated with well washed rat liver mitochondria as described before (33). As shown in Fig. 4A total polysome translation products resolve into a large number of bands (see lane 1). Immunoprecipitation of total translation products with polyclonal antibody to cytochrome P-450c yields a major component of about 54 kDa which comigrates with cytochrome P-450c and a minor species of 57 kDa shown by an

![Fig. 3. Two-dimensional analysis of mitochondrial and microsomal cytochrome P-450. 2.0 µg each of microsomal (A), mitochondrial (B), and a mixture of microsomal and mitochondrial (C) cytochrome P-450 purified by immunoaffinity adsorption were analyzed by the two-dimensional gel system of O'Farrell (29). The proteins were transferred to nitrocellulose membranes, probed with polyclonal antibody to cytochrome P-450c (1:1000 dilution), and developed with the Protoblot immunodetection system as described under "Experimental Procedures." The arrows indicate the top (T) of polyacrylamide-SDS slab gels used for the second dimension. In all three cases the antibody-reactive proteins migrated with an apparent molecular mass of 54 kDa. IEF, isoelectric focusing.](image-url)
antibody suggests that this polyclonal antibody-reactive protein is a precursor which is transported into mitochondria and processed into 54-kDa mature cytochrome P-450 apoprotein.

**DISCUSSION**

It has been demonstrated that varied polycyclic aromatic hydrocarbons cause distinct alterations in mitochondrial structure and function (20, 36–38). Furthermore, a number of studies have shown that structurally diverse carcinogens modify mitochondrial DNA of the target tissues at frequencies severalfold higher than the nuclear DNA (39, 40). Such observations prompted us to look for the presence of cytochrome P-450 in hepatic mitochondria which are capable of activating various polycyclic aromatic hydrocarbons. Recent studies from our laboratory showed that mitoplast preparations from 3-methylcholanthrene-treated rat liver contain cytochrome P-450 isoforms which can activate benzo(a)pyrene under *in vitro* conditions (18, 19). The property of this enzyme was unusual in that its activity could be reconstituted with both adrenodoxin/adrenodoxin reductase as well as NADPH cytochrome P-450 reductase electron transfer systems. Furthermore, the enzyme activity was inhibited by polyclonal antibody to microsomal cytochrome P-450c (19). For these reasons it was essential to determine the molecular properties and the precise cellular location of the cytochrome P-450 isoform associated with the 3-methylcholanthrene-induced mitoplast to rule out the possibility that it may represent contaminating microsomal P-450c. We have addressed this question using a polyclonal antibody which is known to cross-react with both microsomal P-450c and mitochondrial isoform (19) and a monoclonal antibody having exclusive specificity to microsomal P-450c (23).

Experiments on the immunoadsorption of mitochondrial and microsomal cytochrome P-450 to polyclonal antibody raised against nearly homogeneous preparations of cytochrome P-450c (>90% pure) showed that both of the isoforms have a close similarity in size (see Fig. 1). However, three different lines of experiments show that they have inherent differences. First, monoclonal antibody highly specific for cytochrome P-450c does not inhibit the aryl hydrocarbon hydroxylase activity of the mitochondrial cytochrome P-450 in an *in vitro* system reconstituted with bovine adrenodoxin and adrenodoxin reductase (Table I), whereas the polyclonal antibody inhibits the enzyme activity in a concentration-dependent manner. Second, only the polyclonal antibody, but not the monoclonal antibody, cross-reacts with the mitochondrial isoform as tested by the Western blot analysis. Third, resolution on the two-dimensional gels and immunoblot analysis presented in Fig. 3 show that the two isoforms have distinct isoelectric points. These results provide strong support for the possibility that the mitochondrial isoform represents an independent molecular species.

It is now known that almost all of the mitochondrial inner membrane and matrix proteins coded by the nuclear genes are synthesized as precursors with N-terminal extensions and transported across the mitochondrial membranes post-translationally in an energy-dependent manner (41, 42). These extra sequences are removed during the transport by an endoprotease present in the mitochondrial matrix fraction (43). The *in vitro* transport and immunoprecipitation experiments presented in Fig. 4 indeed show that membrane-free polysome translation products contain a polyclonal antibody-reactive 57-kDa protein. In addition a major species of 54 kDa is immunoprecipitated with both polyclonal and monoclonal antibodies. Since it is virtually impossible to purify free pol-

![Fig. 4. In vitro transport of polyclonal antibody-reactive precursor into mitochondria.](image-url)
ysomes completely devoid of the bound forms (35), the 54-kDa species immunoprecipitated from the polysome translation products may be cytochrome P-450c chains. In support of this possibility, only the 54-kDa species, but not the 57-kDa precursor, is immunoprecipitated with monoclonal antibody. Furthermore, isolated mitochondria take up a polyclonal antibody-reactive 54-kDa species and render them protease-resistant. This process is inhibited by uncouplers like valinomycin suggesting that it is a true mitochondrial energy-dependent transport. Furthermore, the species internalized in vitro-incubated mitochondria differ from the 54-kDa species immunoprecipitated from the total polysome translation products by virtue of its inability to cross-react with the monoclonal antibody (Fig. 4B, lanes 7 and 8). These results demonstrate that the polyclonal antibody-reactive cytochrome P-450 detected in the 3-methylcholanthrene-induced rat liver mitoplasts are intramitochondrially located. Since we have been unable to detect significant levels of this cytochrome P-450 in mitoplasts from control liver (Fig. 2A, lane 1), the cytochrome P-450 under study appears to represent a truly inducible type. Experiments are under way to purify this mitochondrial cytochrome P-450 in its biologically active form to further understand its physicochemical and catalytic properties.

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