Purified Liver Microsomal Cytochrome P-450
SEPARATION AND CHARACTERIZATION OF MULTIPLE FORMS*

(Received for publication, February 14, 1975)

DAVID A. HUGGEN; THEODORE A. VAN DER HOEVEN; AND MINOR J. COON

From the Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48104

SUMMARY

During the purification of rabbit liver microsomal cytochrome P-450 (P-450Lm), evidence was obtained for the occurrence of at least four distinct forms. These were distinguished by polyacrylamide gel electrophoresis after treatment with sodium dodecyl sulfate in the presence or absence of mercaptoethanol and were shown to have characteristic spectra as the reduced carbon monoxide complexes. They are designated by their relative electrophoretic mobilities. P-450Lm1, which was purified to apparent homogeneity, is induced by phenobarbital and has a subunit molecular weight of 50,000. P-450Lm4, which was also extensively purified, is induced by β-naphthoflavone and has a molecular weight of 54,000. P-450Lm1,7, which is induced neither by phenobarbital nor β-naphthoflavone, is a mixture of about equal amounts of two forms with molecular weights of 47,000 and 60,000, respectively. Some preparations were obtained containing primarily P-450Lm, or P-450Lm4.

Benzphetamine, ethylmorphine, and β-nitroanisole are hydroxylated preferentially by P-450Lm, and benzpyrene by P-450Lm4. Biphenyl is hydroxylated in both positions 2 and 4 by all of the preparations, but the latter position is strongly favored by the action of P-450Lm4. Testosterone is hydroxylated primarily in position 16α by P-450Lm, and in position 6β by P-450Lm1,7. Although the occurrence of additional forms of the cytochrome with highly similar electrophoretic behavior is not ruled out, it appears that the presence of these forms differing in subunit molecular weight may account for the variety of catalytic activities attributed to this pigment of liver microsomes.

The cytochrome P-450-containing enzyme system of the hepatic endoplasmic reticulum is of particular interest because of its inducibility by many agents and its remarkably broad substrate specificity. Substrates such as fatty acids and steroids, as well as a variety of foreign compounds, including drugs, anesthetics, petroleum products, insecticides, and carcinogens, are hydroxylated or otherwise modified by liver microsomes in the presence of NADPH and molecular oxygen. Several years ago this laboratory reported the solubilization and resolution of the liver microsomal enzyme system into three components: cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine (1-4). The reconstituted enzyme system was shown to catalyze the hydroxylation of a variety of substrates (5, 6). More recently, we have purified cytochrome P-450 to apparent homogeneity from phenobarbital-induced rabbit liver microsomes (7, 8) and have also obtained the reductase in highly purified form (7, 9).

The question of whether the numerous activities attributed to P-450Lm reside in one or more forms of this pigment has been the subject of much investigation. The variable enzyme activities observed in microsomes of animals treated with different inducing agents suggested that numerous forms of P-450Lm might be involved (10-13). On the other hand, kinetic data obtained with microsomal suspensions (14-16) and with the reconstituted enzyme system (5) showed that a number of substrates act as mutually competitive inhibitors, thereby indicating that they may be acted on by a single enzyme. Spectral evidence was reported suggesting the occurrence of two forms of cytochrome P-450 in liver microsomes, induced either by phenobarbital or polycyclic aromatic hydrocarbons (17-21). The genetic regulation also supported the involvement of a separate enzyme for aryl hydrocarbon hydroxylation (22). Fractionation procedures have been applied to liver microsomes following the administration of different inducing agents to animals (23), and it was shown that substrate specificity resides in the different cytochrome fractions as tested in reconstituted systems (24-26). In the present paper we describe the separation and purification of multiple forms of cytochrome P-450 from rabbit liver microsomes and their characterization as distinct proteins.

Liver microsomes were prepared as described previously (7) from rabbits treated with phenobarbital (8) or β-naphthoflavone (26), except that the microsomal pellets were frozen overnight before treatment with pyrophosphate buffer and sonication was omitted. This procedure and subsequent purification steps were carried out at 0-4°C, and the preparations were stored at -20°C. NADPH-cytochrome P-450 reductase was partially purified from rabbit liver microsomes (7, 9). Protein concentrations were determined by the procedure of Lowry et al. as already described (7, 27). All buffers were at pH 7.4 and contained 20% glycerol. Hydroxylapatite, type B (28), was mixed with an equal weight of Whatman CF1 cellulose powder.

Separation and Purification of Different Forms of Cytochrome P-450 from Phenobarbital-induced Microsomes—The pyrophosphate-extracted microsomal suspension was solubilized with cholate, fractionated with polyethylene glycol, and applied to a DEAE-cellulose column in the presence of Renex-690 as previously described (7). Tris-acetate buffer, 0.01 M, containing 1.0 mM EDTA, 0.1 mM diethiothreitol, and 0.5% Renex, was then passed through the column until no more cytochrome was eluted. A second cytochrome eluate was obtained by washing the column

* This research was supported by Grant BMS71-01195 from the National Science Foundation and Grant AM 10889 from the United States Public Health Service.

† Postdoctoral Fellow, United States Public Health Service.

‡ Present address, Department of Medicinal Chemistry. University of Maryland, Baltimore, Maryland 21201.
with the same buffer solution to which 0.05 M KCl had been added. The first eluate from DEAE-cellulose was applied to a hydroxylapatite-cellulose column previously equilibrated with 0.01 M potassium phosphate buffer containing 0.1 mM dithiothreitol and 0.1% Renex. The column was washed with the same buffer solution and then eluted with 0.05 M phosphate buffer containing 0.1 mM dithiothreitol and 0.1% Renex until no more cytochrome was eluted. Elution first with a similar solution containing 0.1 M phosphate and then 0.3 M phosphate was carried out in a similar fashion. The 0.05 and 0.3 M phosphate eluates were treated individually with calcium phosphate gel (7) to concentrate the protein and reduce the detergent concentration and were then dialyzed overnight against 0.01 M phosphate buffer containing 1.0 mM EDTA. The resulting preparations are designated Fractions A and B, respectively.

The second eluate from DEAE-cellulose was similarly treated with calcium phosphate gel and dialyzed. This preparation is designated Fraction C.

**Purification of Cytochrome P-450 from β-Naphthoflavone-induced Microsomes**—Liver microsomes from β-naphthoflavone-induced animals were extracted with pyrophosphate, solubilized, and treated with polyethylene glycol (7). The 5 to 10% and 10 to 13% polyethylene glycol precipitates were combined and further purified by DEAE-cellulose column chromatography (7). The cytochrome fraction eluted by the 0.01 M Tris buffer solution was applied to a hydroxylapatite-cellulose column as described above. The column was washed with 0.01 M phosphate buffer containing 0.1 mM dithiothreitol and 0.1% Renex and eluted with a similar buffer solution containing 0.1 M phosphate and then with one containing 0.3 M phosphate. The latter eluate was treated with calcium phosphate gel or Amberlite XAD-2 as described earlier (7) and was dialyzed against 0.01 M phosphate buffer containing 1.0 mM EDTA. This preparation, called Fraction D, became slightly turbid during dialysis and storage after treatment with calcium phosphate, but when treated with Amberlite XAD-2 instead it remained soluble, possibly because a small amount of detergent was still present. All of the other cytochrome P-450 fractions remained soluble during storage for several months.

**Electrophoresis of P-450LM Fractions**—The proteins in normal and phenobarbital-induced liver microsomes and in the various purified fractions were treated with sodium dodecyl sulfate and mercaptoethanol and submitted to discontinuous polyacrylamide gel electrophoresis, as shown in Fig. 1. The poly peptide patterns revealed by staining with Coomassie blue are highly reproducible in this system. Beginning with the major microsomal band of greatest electrophoretic mobility, the bands are numbered according to decreasing mobility and increasing molecular weight. The P-450LM in Fraction A, which has been purified to apparent homogeneity (8), clearly corresponds to the phenobarbital-inducible band (Band 2) in liver microsomes. Fraction B contains primarily Band 4, as well as a smaller amount of Band 2, whereas Fraction C contains Bands 1 and 7 as the major components along with lesser amounts of the intermediate bands. In other experiments not presented, preparations have been obtained containing primarily P-450LM, or P-450LM, Fraction D contains Band 4, which corresponds to the β-naphthoflavone-inducible band. Fig. 2 shows the electrophoretic results obtained when sodium dodecyl sulfate treatment was carried out in the absence of mercaptoethanol, thereby permitting the detection of heme-containing peptides. Under these conditions the resolution is less satisfactory than that in the discontinuous system. Although some free heme was liberated under these conditions and migrated rapidly to the bottom of the gels, a single heme peptide was detected in Fractions A, B, and D corresponding closely to the bands seen by staining with Coomassie blue. In Fraction C, heme peptides were detected corresponding to Bands 1 and 7 as well as to two bands of intermediate molecular weight. These results indicate that the proteins which have been separated and purified to varying extents from liver microsomes are indeed different hemeproteins. It should be noted that multiple bands apparently corresponding to different forms of cytochrome P-450 have been reported by others from electrophoresis of the proteins in rat liver microsomal suspensions (30-32).

**Physical Properties and Catalytic Activity of Different Forms of P-450LM**—Table I summarizes the extent of purification and some of the properties of the forms of P-450LM in the various fractions. The different forms are designated by their electrophoretic behavior, P-450LM, being the phenobarbital-inducible form, P-450LM, the β-naphthoflavone-inducible form, etc. The apparent molecular weights of the polypeptide chains, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with standard proteins, range from 47,000 to 60,000 (33). The absorption maxima of the reduced CO complexes are also significantly different. The catalytic activities of the various P-450LM fractions toward various substrates are given in Table II. Some striking differences are obvious, but the results need to be interpreted with caution since all of the individual forms have not yet been fully purified and separated. P-450LM has clearly the most effective toward benzphetamine, ethylmorphine, and p-
FIG. 2. Separation of heme peptides by electrophoresis on polyacrylamide gel in a continuous buffer system. The enzyme preparations were treated with sodium dodecyl sulfate at 5°C in the absence of mercaptoethanol and then submitted to electrophoresis and stained as described by Welton and Aust (30), except that acetate buffer was used for heme staining. For each preparation the following are shown, from left to right: photograph of a gel stained for protein with Coomassie blue, photograph of a gel stained for heme, and a diagram of the latter gel.

TABLE I

Properties of different forms of cytochrome P-450 from rabbit liver microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Agent administered to animals</th>
<th>Electrophoretic identification</th>
<th>Apparent molecular weight*</th>
<th>Absorption maximum of reduced CO complex**</th>
<th>Specific content of cytochrome P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Phenobarbital</td>
<td>LM2</td>
<td>50,000</td>
<td>451.0</td>
<td>18.0</td>
</tr>
<tr>
<td>B</td>
<td>Phenobarbital</td>
<td>LM4</td>
<td>54,000</td>
<td>448.0</td>
<td>13.6</td>
</tr>
<tr>
<td>C</td>
<td>Phenobarbital</td>
<td>LM4,2</td>
<td>66,000</td>
<td>445.8</td>
<td>3.3</td>
</tr>
<tr>
<td>D</td>
<td>β-Naphthoflavone</td>
<td>LM4</td>
<td>54,000</td>
<td>445.7</td>
<td>11.7</td>
</tr>
</tbody>
</table>

*Estimated by polyacrylamide gel electrophoresis with standardization by proteins of known molecular weight.
**In repeated experiments the variation in these values was within 0.3 nm in a calibrated Cary 14 spectrophotometer.

TABLE II

Substrate specificity of different forms of cytochrome P-450

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Position of hydroxyl group in product</th>
<th>Activity of P-450LM preparation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzphetamine</td>
<td>LM1 66, LM2 3.0, LM3 7.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Ethylmorphine</td>
<td>LM1 6.1, LM2 3.0, LM3 3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Ethylmorphine</td>
<td>LM3 6.4, LM4 0.8, LM5 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>p-Nitroanisole</td>
<td>LM1 1.0, LM2 0.7, LM3 0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>p-Nitroanisole</td>
<td>LM4 0.7, LM5 0.4, LM6 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>p-Nitroanisole</td>
<td>LM7 0.43, LM8 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Testosterone</td>
<td>LM1 0.04, LM2 0.02</td>
<td>Trace</td>
</tr>
<tr>
<td>Testosterone</td>
<td>LM3 0.04, LM4 0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Testosterone</td>
<td>LM5 0.02, LM6 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Testosterone</td>
<td>LM7 0.02, LM8 0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Testosterone</td>
<td>LM9 0.02, LM10 0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Expressed as nanomoles of product formed per min per nmol of cytochrome P-450.
**Fraction B.
***Fraction D.
****These values were at the minimal level of detection.
*****We are indebted to Dr. Daniel W. Nebert of the National Institutes of Health for these determinations.

The reaction mixtures were incubated at 30°C and contained, per ml, P-450LM (0.1 nmol), NADPH-cytochrome P-450 reductase (specific activity, 0.1 nmol of cytochrome c reduced per min per mg of protein; 16 µg), dihydroxyeugenyl-3-phosphorylcholine (30 µg), deoxycholate (50 µg), Hepes buffer, pH 7.4 (50 mmol), MgCl2 (15 mmol), substrate (1.0 nmol of benzphetamine, p-nitroanisole, or biphenyl; 0.12 Mmol of testosterone; 10 µmol of ethylmorphine; 0.25 µmol of aniline or 0.08 µmol of benzyrene), and 0.4 µmol of NADPH as the last addition. These conditions were shown to be optimal for the most active substrate, benzphetamine, with this P-450LM preparation and are typical of those used in previous studies in this laboratory with the reconstituted system (5, 7). NADPH was omitted in control experiments. The hydroxylation of the substrates was determined as follows: benzphetamine and ethylmorphine by formaldehyde formation (34), p-nitroanisole by p-nitrophenol formation (35), [14C]aniline by the radioactivity of p-aminophenol (36), biphenyl by fluorometric assay of the products (37), [14C]testosterone by the radioactivity of the products after separation by paper chromatography (38), and benzyrene by a fluorometric procedure (39).

These results show that the various forms of cytochrome P-450 which have been separated and purified to different extents from rabbit liver microsomes differ in their physical and catalytic properties. Variations in the level of as many as four forms of P-450LM due to different types of induction, the age and sex of the animals, etc., could account for the differences in catalytic

nitroanisole. Biphenyl is hydroxylated in both positions 2 and 4 by all fractions, but the activity toward the latter position is predominant with P-450LM. The positional specificity toward testosterone is also shown with P-450LM most effective in producing the 1α isomer and LM3, the 6β isomer of the hydroxylated product. Some activity toward benzphetamine was obtained in all fractions, but P-450LM was most active. These results show that the various forms of cytochrome P-450 which have been separated and purified to different extents from rabbit liver microsomes differ in their physical and catalytic properties. Variations in the level of as many as four forms of P-450LM due to different types of induction, the age and sex of the animals, etc., could account for the differences in catalytic
activity observed by various investigators using microsomal suspensions. On the other hand, the occurrence of additional forms not distinguishable by electrophoresis cannot yet be ruled out. The individual forms of P-450 may have the ability to bind many or all of the potential substrates but differ in their relative efficiency in the hydroxylation of such compounds.

REFERENCES
Purified liver microsomal cytochrome P-450. Separation and characterization of multiple forms.
D A Haugen, T A van der Hoeven and M J Coon


Access the most updated version of this article at http://www.jbc.org/content/250/9/3567

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/9/3567.full.html#ref-list-1