# CYTOCHROME P-450 PURIFIED TO APPARENT HOMOGENEITY FROM PHENOBARBITAL-INDUCED RABBIT LIVER MICROSOMES: CATALYTIC ACTIVITY AND OTHER PROPERTIES*

Theodore A. van der Hoeven, David A. Haugen,† and Minor J. Coon

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

Received July 30,1974

Summary: Cytochrome P-450 was purified to a content of over 17 nmoles per mg of protein from liver microsomes of phenobarbital-treated rabbits by fractionation with polyethylene glycol 6000, DEAE-cellulose column chromatography, and hydroxylapatite column chromatography in the presence of Renex 690, a nonionic detergent. The purified preparation exhibited a single polypeptide band (molecular weight, 49,000 daltons) when submitted to SDS-polyacrylamide gel electrophoresis. Cytochromes P-420 and $b_5$ and NADPH-cytochrome c reductase were absent. The reconstituted system containing purified cytochrome P-450, reductase, and phosphatidylcholine catalyzed the hydroxylation of benzphetamine, cyclohexane, aniline, and laurate.

The solubilization and resolution of the liver microsomal enzyme system containing cytochrome P-450 was reported by this laboratory in 1968 (1). NADPH-cytochrome P-450 reductase (2) and phosphatidycholine (3) were shown to be required as well as cytochrome P-450 in the reconstituted system for the hydroxylation of a variety of substrates. Although the cytochrome P-450 of liver microsomes ($P\text{-}450_{LM}$) has proved to be an unusually difficult protein to purify, the use of ionic and nonionic detergents along with suitable protective agents has resulted in significant progress. Procedures have been reported by several laboratories for the partial purification of $P\text{-}450_{LM}$ (4-10), and we have recently obtained this cytochrome at a concentration of 13 to 15 nmoles per mg of protein (11). The present communication describes the further purification of $P\text{-}450_{LM}$ to an apparently homogeneous state and the catalytic activity and other properties of such preparations.

Purification of $P\text{-}450_{LM}$: The methods used for the purification of $P\text{-}450_{LM}$ from pyrophosphate-treated microsomes are summarized in Table I. New Zealand male rabbits were allowed to drink a 0.1% phenobarbital solution for five days

* This research was supported by Grant AM-10339 from the United States Public Health Service and Grant GB-30419X from the National Science Foundation.
† Postdoctoral Fellow, United States Public Health Service.

Table I

Purification of liver microsomal cytochrome P-450

| Preparation | Protein (mg) | Cytochrome P-450 content (nmoles/mg protein) | Yield (%) |
|---|---|---|---|
| Pyrophosphate-treated, sonicated microsomes | 3,290 | 3.1 (2.6-3.6) | 100 |
| Polyethylene glycol precipitate | 1,530 | 5.9 (5.4-7.0) | 88 |
| DEAE-cellulose column eluate | 181 | 13.7 (9.0-15.2) | 24 |
| Hydroxylapatite column eluate | 18.5 | 17.4 (13.0-17.4) | 3 |

The numbers in parentheses indicate the range of concentrations found in a series of such purifications. Cytochrome P-450 was determined as the reduced CO complex (12).

and were fasted the last day. The isolation and extraction of the liver microsomes, fractionation with polyethylene glycol 6000, and DEAE-cellulose column chromatography followed by elution from calcium phosphate gel were carried out essentially as described elsewhere (11) except that a Bio-Gel P-60 concentration step was omitted. The following procedures were carried out at 4°, and protein concentrations were determined according to Lowry et al. (13). All buffer solutions used in the purification procedure were at pH 7.4 and contained 20% glycerol.

The preparation fractionated on DEAE-cellulose and treated with calcium phosphate gel (11) was dialyzed overnight against 10 volumes of 0.01 M phosphate buffer containing 1.0 mM EDTA and diluted with the same buffer mixture to a protein concentration of 1.0 mg per ml. Renex 690 (polyoxyethylenealkylaryl ether), a nonionic detergent, was added to a concentration of 0.1% ($^{v}/_{v}$), and the enzyme solution was applied to a column of hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories; 2.5 X 11 cm) previously equilibrated with 0.01 M phosphate buffer containing 0.1% Renex (hereafter called phosphate-Renex). The column was washed with 250 ml of 0.01 M phosphate-Renex containing 0.1 M KCl, and the protein solution eluted from the column with 250 ml of 0.1 M phosphate-Renex was diluted with an equal volume of water. Calcium phosphate gel prepared according to Keilin and Hartree (14) (200 mg in 10 ml) was added, and the mixture was stirred for 15 min and cen-

trifuged at 4,000 X g for 5 min. The reddish pellet was suspended in 250 ml of 0.01 M phosphate buffer, and the mixture was stirred for 15 min. The pellet was recovered, washed again in the same manner, and eluted by suspension in 25 ml of 0.3 M phosphate buffer with stirring for 15 min. The elution step was repeated, and the supernatant solutions obtained upon centrifugation were combined and dialyzed against 10 volumes of 0.01 M phosphate buffer. The red solution was diluted to a protein concentration of 0.5 mg per ml, Renex was added to a concentration of 0.1%, and the mixture was again submitted to column chromatography on hydroxylapatite as described above. Fractions eluted by 500 ml of 0.05 M phosphate-Renex were combined and diluted with an equal volume of water. A calcium phosphate suspension (200 mg in about 10 ml) was added, and the mixture was stirred for 15 min and then centrifuged. The pellet was washed and eluted once as described above. The resulting P-450$_{LM}$ solution was dialyzed overnight against 20 volumes of 0.01 M phosphate buffer and stored at -20°. A second elution improved the yield but gave a preparation of somewhat lower P-450 content.

Properties of Purified P-450$_{LM}$: As shown in Fig. 1, SDS-polyacrylamide gel electrophoresis was used to determine the purity of the P-450$_{LM}$ and its relationship to the various polypeptides present in microsomes. The cytochrome purified by column chromatography on hydroxylapatite to a concentration of 17.4 nmoles per mg of protein exhibited a single band at each of the two concentrations tested. Such results are indicative of homogeneity but do not rule out the presence of apocytochrome P-450 or of other polypeptides of identical molecular weight. In contrast, less purified preparations contained more than one component (11). For comparison, the multiple bands seen by SDS-polyacrylamide electrophoresis of the proteins in liver microsomes from normal and phenobarbital-treated animals are also shown. The results strongly indicate that the band which is sharply increased in intensity by drug treatment (at 6.9 cm on the adjacent scale) corresponds to the cytochrome P-450 isolated by the purification procedures described. In other electrophoretic experiments proteins of known molecular weight were included as standards, and the purified P-450$_{LM}$ was shown to have a molecular weight of about 49,000.

No cytochrome P-420, cytochrome $b_5$, NADPH-cytochrome P-450 reductase (measured by its activity toward cytochrome c), or NADH-cytochrome $b_5$ reductase (measured by its activity toward ferricyanide) could be detected in the purified P-450$_{LM}$. The absolute spectra of the oxidized and reduced forms of the purified protein shown in Fig. 2 are similar to those reported earlier for the partially purified form (11). However, the reduced CO

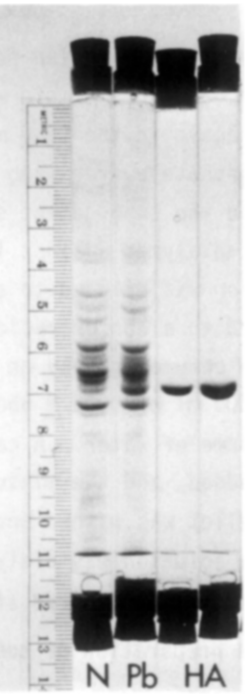


Fig. 1. Electrophoretic homogeneity of purified P-450$_{LM}$. The enzyme preparations were treated with SDS and mercaptoethanol and submitted to polyacrylamide gel electrophoresis by a slight modification of the method of Laemmli (15) with a 7.5% separating gel. Migration was from top to bottom, and the position of the tracking dye was marked by a stainless steel wire. The gels were fixed in 5:1:4 methanol-acetic acid-water and then stained with 0.01% Coomassie Blue R250 in the same solvent mixture. The following samples were analyzed at the protein level indicated: N, 15 µg; Pb, microsomes from phenobarbital-induced animals, 15 µg; and HA, P-450$_{LM}$ (17.4 nmoles per mg of protein) eluted from hydroxylapatite, 2.5 and 7.5 µg.

complex has a maximum at 452 nm, whereas in less purified preparations the maximum is at 450 nm. In other experiments typical Type I difference spectra were obtained upon the addition of benzphetamine and cyclohexane.

The activity of various substrates in the reconstituted enzyme system containing purified P-450$_{LM}$ as the rate-limiting component, along with highly purified detergent-solubilized NADPH-cytochrome P-450 reductase and dilauroylglyceryl-3-phosphorylcholine as the source of phospholipid, are presented in Table II. The turnover numbers for benzphetamine, cyclohexane, and aniline are several times as great as ordinarily obtained with intact microsomes, whereas the number for laurate is about half as great as in microsomes. Clearly, the purified P-450$_{LM}$ retains the ability to catalyze the hydroxylation of a variety of substrates. Cytochrome $b_5$ obviously plays

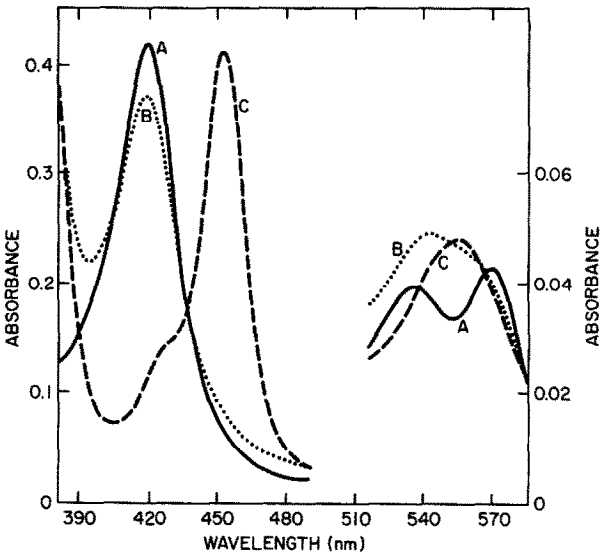


Fig. 2. Absolute spectra of purified cytochrome P-450 diluted to a concentration of 3.5 nmoles per ml in 0.05 M phosphate buffer, pH 7.4, containing 20% glycerol and 1.0 mM EDTA: A, oxidized; B, dithionite-reduced; C, dithionite-reduced CO complex. The spectrum at higher wavelengths is shown in the inset with a 5-fold expanded scale.

Table II

Substrate hydroxylation in reconstituted enzyme system containing purified P-450$_{LM}$

| Substrate | Turnover number |
|---|---|
| Benzphetamine | 59 |
| Cyclohexane | 59 |
| Aniline | 2.8 |
| Laurate | 0.8 |

The turnover numbers are expressed as moles of product formed per min per mole of cytochrome P-450 at 30°. The reaction mixtures contained cytochrome P-450 (0.1 nmole) as the rate-limiting component along with purified rabbit liver microsomal NADPH-cytochrome P-450 reductase (specific activity, 20 µmoles of cytochrome c reduced per min per mg of protein; 18 µg), dilauroylglyceryl-3-phosphorylcholine (30 µg), deoxycholate (100 µg), Hepes buffer, pH 7.4 (50 µmoles), $MgCl_2$ (15 µmoles), and NADPH (0.1 µmole) as the final addition. Benzphetamine demethylation was determined by formaldehyde formation (16,17), cyclohexane hydroxylation by the rate of NADPH disappearance at 340 nm (18), aniline hydroxylation by p-aminophenol formation (19,20), and laurate hydroxylation by a radioactive assay (21).

no direct role in substrate hydroxylation, since no detectable amounts of this cytochrome were present in the purified P-450$_{LM}$ and reductase used in the reconstituted system. On the other hand, a facilitating role of cytochrome $b_5$ in electron transfer in microsomal membranes is not ruled out.

Liver microsomes from phenobarbital-treated rabbits have recently been shown to contain, in addition to the cytochrome P-450 which has been purified and characterized as described in the present communication, at least two other forms (22). These have been partially separated and shown to differ in molecular weight as well as in their spectral and catalytic properties. Such findings indicate that multiple forms of P-450$_{LM}$ probably account for the remarkably broad spectrum of activities attributed to this pigment in microsomal membranes.

Acknowledgement: We are grateful to Joanne K. Heidema, Barbara M. Michniewicz, and Sylvia B. Dahl for technical assistance.

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