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PURIFICATION AND PROPERTIES OF P-450LM$_{3b}$, A CONSTITUTIVE FORM OF CYTOCHROME P-450, FROM RABBIT LIVER MICROSOMES

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SUMMARY: This laboratory has previously reported the occurrence in rabbit liver microsomes of a non-inducible form of cytochrome P-450, designated P-450LM$_{3b}$ because of its electrophoretic mobility relative to that of phenobarbital-inducible P-450LM$_2$ and 5,6-benzoflavone-inducible P-450LM$_4$. In the present study, P-450LM$_{3b}$ was purified to electrophoretic homogeneity and a specific content of over 19 nmol per mg of protein by chromatographic procedures carried out in the presence of detergents. The isolated cytochrome has a minimal molecular weight of 52,000 and exhibits absorption maxima at 418, 537, and 571 nm in the oxidized state, 412 and 547 nm in the reduced state, and 451 and 555 nm as the CO complex. In a reconstituted system containing NADPH-cytochrome P-450 reductase and phosphatidylcholine, P-450LM$_{3b}$ has relatively high activity in the hydroxylation of testosterone in the 6β and 16α positions as well as significant activity toward a number of other substrates tested. The NADPH oxidase activity of P-450LM$_{3b}$ is less than half that of P-450LM$_2$ and LM$_4$.

Following the solubilization and resolution of the cytochrome P-450-containing enzyme system of liver microsomes into three components (P-450LM,[1] NADPH-cytochrome P-450 reductase, and phosphatidylcholine) and reconstitution of those components into an active hydroxylating complex (2-4), this laboratory reported the separation and characterization of multiple forms of the cytochrome from rabbit liver microsomes (5). The various isozymes were shown to differ in their electrophoretic behavior, subunit molecular weights, and spectral characteristics, and to have somewhat different but overlapping substrate specificities. The two major forms of the cytochrome in rabbit liver microsomes, both of which are inducible, were first obtained in a highly purified state some years ago: P-450LM$_2$ (6-9) and P-450LM$_4$ (5,10,11).

The present paper is concerned with the isolation and characterization of P-450LM$_{3b}$, so designated because it is one of two proteins in rabbit liver microsomes with mobility intermediate between that of P-450LM$_2$ and LM$_4$ upon

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[1]The abbreviations used are: P-450LM, liver microsomal cytochrome P-450; and dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine. The various forms of P-450LM are numbered according to their relative electrophoretic mobilities in agreement with the general recommendation of the commission on Biochemical Nomenclature for isozymes (2).

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SDS-polyacrylamide gel electrophoresis. This enzyme was partially purified earlier as a by-product of P-450$_{LM_2}$ isolation (9,12,13) and shown to be active in the hydroxylation of benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol (14) as well as warfarin (15).

MATERIALS AND METHODS

NADPH-cytochrome P-450 reductase prepared from rabbit liver microsomes as described elsewhere (16) was electrophoretically homogeneous and had a specific activity of 44 μmol of cytochrome c reduced per min per mg of protein. Hydroxylapatite (Bio-Gel HTP) and Cellex-P (0.93 meq per g) were obtained from Bio-Rad and DEAE-Sepharose CL-6B from Pharmacia. d-Benzphetamine was kindly furnished by Dr. Paul W. O'Connell of the Upjohn Co. and Renex 690 by ICI America. [4-^{14}C]Testosterone obtained from New England Nuclear was diluted to a specific activity of 8.3 μCi per μmol with the unlabeled compound. The 6β, 7α, and 16α hydroxy derivatives were separated by thin layer chromatography according to the method of Orton and Philpot (17)[2] after addition of unlabeled standards, and the resulting samples were scraped into scintillation vials for radioactivity determination. The demethylation of benzphetamine, ethylmorphine, aminopyrine, and chlorcyclizine was determined by formaldehyde formation (18). All assays were carried out with the cytochrome as the limiting component under conditions described previously (5). Protein concentrations were measured according to Lowry et al. (19) as modified by Bensadoun and Weinstein (20). Other methods were as described previously (6).

RESULTS AND DISCUSSION

Purification of P-450$_{LM_{3b}}$: The procedures used for the purification of P-450$_{LM_{3b}}$ from pyrophosphate-treated microsomes from uninduced New Zealand male rabbits are summarized in Table I. Hepatic microsomes were isolated, solubilized with cholate, and fractionated with polyethylene glycol 6000 as described elsewhere (13) except that the 8 to 10% and 10 to 12% polyethylene glycol precipitates were suspended in 10 mM potassium phosphate, pH 6.0, containing 20% glycerol, 1 mM EDTA, and 0.5% Renex 690 (Buffer A) and dialyzed overnight against the same buffer. Unless otherwise indicated, all buffers contained glycerol, EDTA and Renex at these concentrations and the operations were conducted at 4°.

Either the 8 to 10% or 10 to 12% fraction from polyethylene glycol precipitation was used for subsequent purification steps, but the latter fraction gave a higher yield and purity after the first chromatography step. The molar ratio of Renex to P-450$_{LM}$ in the 10 to 12% fraction was adjusted to 1,000:1 by addition of a 10% (w/v) solution of the detergent. This ratio of detergent to hemeprotein has been shown to yield a less aggregated form of the hemeprotein

[2]Personal communication from Dr. R. M. Philpot.

TABLE I

Purification of Liver Microsomal Cytochrome P-450$_{LM_{3b}}$

Preparation	Protein (mg)	Cytochrome P-450 content (nmol/mg protein)	Yield[a] (%)
Pyrophosphate-treated microsomes	3,060	2.1	100
Polyethylene glycol precipitate (10 to 12%)	714	3.6	40
Cellex-P column eluate; calcium phosphate gel eluate	43	6.9	5
DEAE-Sepharose column eluate	6.8	12.2	1.3
Hydroxylapatite column eluate	1.8	19.2	0.5

[a]Calculated on the basis of all forms of P-450$_{LM}$ present in microsomes.

(21). The solution was applied to a Cellex-P column (2.7 X 30 cm) previously equilibrated with Buffer A, followed by one liter of the same buffer. This step removed cytochrome b_5 and the small amount of NADPH-cytochrome P-450 reductase in the polyethylene glycol fraction. The column was treated with one liter of 50 mM Buffer A and then with 100 mM Buffer A to yield a fraction enriched in P-450$_{LM_{3b}}$. This solution was treated with Amberlite XAD-2, adsorbed onto calcium phosphate gel, eluted with a small volume of 0.3 M phosphate buffer, pH 7.7, and dialyzed against 10 mM phosphate buffer at the same pH without Renex present (Buffer B). The resulting preparation (containing about 1.0 mg of protein per ml) was then brought to 0.5% and 1.25% in Renex and sodium cholate, respectively, and layered onto a DEAE-Sepharose column (1.5 X 20 cm) equilibrated with Buffer B containing 0.5% Renex. The column was washed with 250 ml of the equilibrating buffer, which caused a narrow red band to move approximately half way down the column. This band was eluted with 250 ml of 20 mM Buffer B, and the preparation was treated with Amberlite. An equal volume of 20% glycerol was added, and the solution was applied to a hydroxylapatite column (1.0 X 4.0 cm) previously equilibrated with 10 mM phosphate buffer, pH 7.7, containing 0.1 mM EDTA but no Renex (Buffer C). The column was washed with the same buffer until the absorbance of the eluate was less than 0.01 at 276 nm, which is the absorbance maximum for Renex 690, and treated successively with 80 mM Buffer C, 80 mM Buffer C containing 0.2% sodium cholate, and finally with 200 mM Buffer C containing 0.2% sodium cholate, which eluted P-450$_{LM_{3b}}$. The purified cytochrome was dialyzed against 10 mM phosphate buffer, pH 7.7, and stored at -20°.

Properties of Purified P-450$_{LM_{3b}}$: As shown in Fig. 1, SDS-polyacrylamide gel electrophoresis was used to determine the purity of P-450$_{LM_{3b}}$ and its mobility compared to that of P-450$_{LM_2}$ and P-450$_{LM_4}$. P-450$_{LM_{3b}}$ purified

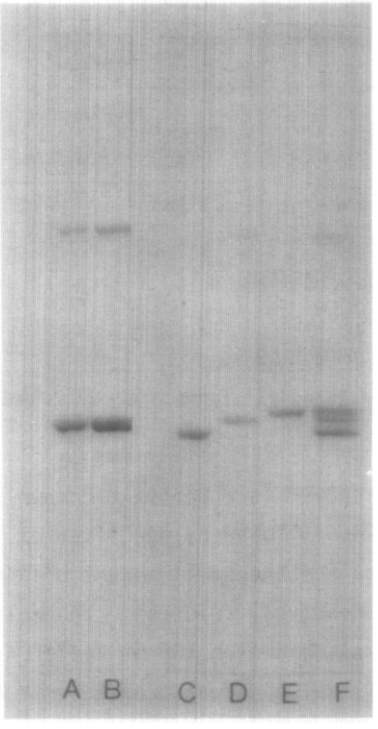

Fig. 1. Electrophoretic behavior of purified P-450$_{LM_{3b}}$. The enzyme preparations were treated with SDS and mercaptoethanol and submitted to polyacrylamide slab gel electrophoresis by a slight modification of the method of Laemmli (21) with a 7.5% separating gel. Migration was from top to bottom. The gel was stained with 0.01% Coomassie Blue R250. The samples were analyzed at the protein levels indicated: A and B, LM_{3b}: 0.7 and 1.5 µg, respectively; C, LM_2: 0.5 µg; D, LM_{3b}: 0.5 µg; E, LM_4: 0.5 µg; and F, a mixture of LM_2, LM_{3b} and LM_4: 0.5 µg each.

through hydroxylapatite column chromatography, with a specific content of 17 nmol per mg of protein, exhibited a single major polypeptide band. The only contaminant was a minor band of higher molecular weight believed to be a dimer of LM_{3b}; LM_2 and LM_4 also show some dimer formation. In other experiments, proteins of known molecular weight were included as standards, and purified P-450$_{LM_{3b}}$ was found to have a subunit molecular weight of 52,000. This compares with 49,200 and 53,000 determined for P-450$_{LM_2}$ and P-450$_{LM_4}$, respectively. As can be seen in the figure, P-450$_{LM_{3b}}$ is clearly distinct electrophoretically from the two major isozymes of P-450$_{LM}$.

The absorption spectra of the purified cytochrome in the oxidized and reduced forms and as the CO complex are given in Fig. 2. The oxidized spectrum is that of a low spin hemeprotein resembling P-450$_{LM_2}$ but not P-450$_{LM_4}$, which is isolated largely in the high spin state (9). The absorption maxima

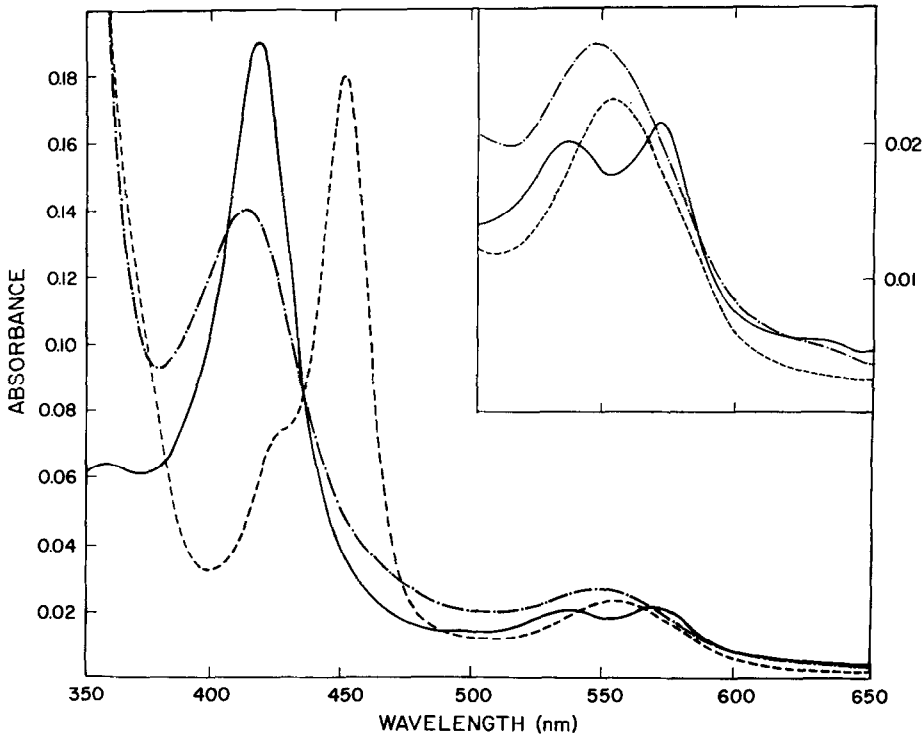

Fig. 2. Absolute spectra of purified P-450$_{LM_{3b}}$ at a concentration of 1.7 μM in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA: (——), oxidized; (-·-), reduced; and (– –), dithionite-reduced CO complex.

of P-450$_{LM_{3b}}$ are as follows: oxidized form, 418, 537, and 571 nm; reduced form, 412 and 547 nm; and CO complex, 451 and 555 nm.

Table II shows the activity of the purified cytochrome with a series of substrates. P-450$_{LM_{3b}}$ exhibited low but significant activity with all of the drugs tested; less purified preparations have previously been shown to catalyze the hydroxylation of warfarin (15) and of both benzo[a]pyrene and its 7,8-dihydrodiol (14). Of particular interest, P-450$_{LM_{3b}}$ has a much higher relative activity in testosterone hydroxylation in the 6β and 16α positions than do the other isozymes. In other experiments the NADPH oxidase turnover number of P-450$_{LM_{3b}}$ (determined in the absence of substrate) was 15, which is less than half the corresponding values for P-450$_{LM_2}$ and $_{LM_4}$.

Ingelman-Sundberg *et al.* (23,24) have recently described the purification of P-450$_{LM_3}$ from phenobarbital-induced animals by an extension of our procedure for P-450$_{LM_2}$ and reported that their preparation catalyzes the hydroxylation of androstenedione at a higher rate in the presence of

TABLE II

Substrate Hydroxylation in Reconstituted Enzyme System Containing Purified Isozymes of P-450$_{LM}$

Substrate	Activity (nmol/min/nmol P-450$_{LM}$)		
	LM_2	LM_{3b}	LM_4
Benzphetamine	41	5.9	6.4
Aminopyrine	7.7	9.7	10
Chlorcyclizine	1.5	6.4	1.0
Ethylmorphine	4.6	4.9	2.6
Testosterone, 6β	0.10	1.10	0.21
" 7α	<0.02	<0.02	<0.02
" 16α	0.10	0.45	<0.02

The reaction mixtures contained P-450$_{LM_2}$ (0.1 nmol), P-450$_{LM_4}$ (0.2 nmol), or P-450$_{LM_{3b}}$ (0.2 nmol), NADPH-cytochrome P-450 reductase (at a molar concentration 10% greater than that of P-450$_{LM}$), dilauroyl-GPC (30 μg), Tris-chloride buffer, pH 7.5 (150 μmol), the drug substrate (1 μmol), and NADPH (1 μmol) as the last addition in a final volume of 1.0 ml and were incubated at 30°.

With testosterone (0.31 μmol) the conditions were the same, except that the mixtures contained $MgCl_2$ (0.75 μmol) and a higher level of P-450$_{LM}$ (1 nmol) and the incubation was at 37°. Turnover numbers of 0.02 and below are not considered significant.

reconstituted phospholipid vesicles. In studies in press, Miki *et al.* (25) have indicated that a form of cytochrome P-450 with a high affinity for cytochrome b_5 was isolated with the aid of chromatography on a column of immobilized cytochrome b_5 and apparently corresponds to our P-450$_{LM_{3b}}$, and Johnson (26) has reported the electrophoretic properties and immunoreactivity of "form 3". It needs to be determined with certainty whether the purified rabbit liver microsomal enzyme preparations obtained in those laboratories contain P-450$_{LM_{3a}}$, $_{3b}$, or both, before we can conclude whether they represent the same cytochrome as our P-450$_{LM_{3b}}$. Such information is particularly important in view of the remarkably broad specificity of these catalysts and the practice of different laboratories of testing different substrates. An important question to which we have as yet only a partial answer because of limited data is whether the inducible forms of P-450$_{LM}$ are involved primarily in xenobiotic metabolism and the non-inducible forms play a more important role in the metabolism of physiologically important lipids. The present paper and the work of Ingelman-Sundberg with androstenedione provide suggestive evidence for this concept.

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