Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1020-1026

PURIFICATION OF CYTOCHROME P-450 FROM LIVER MICROSOMES OF PHENOBARBITAL-TREATED GUINEA PIGS

Mitsukazu Kitada, Chiyo Yamazaki, Kouzi Hirota and Haruo Kitagawa

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba, Japan

Received March 6, 1980

SUMMARY: Cytochrome P-450 was purified from phenobarbital-treated guinea pigs to a specific content of 19.8 nmoles per mg of protein, and was free of cytochrome b_5 and NADPH-cytochrome c reductase. The purified cytochrome P-450 gave a single protein band on sodium dodecylsulfate-polyacrylamide gel electrophoresis, and an apparent molecular weight of about 49,000 was estimated. Benzphetamine N-demethylation activity could be reconstituted by mixing the purified cytochrome, NADPH-cytochrome c reductase and phosphatidylcholine.

It has been well known that there are qualitative and quantitative species differences in hepatic microsomal drug hydroxylase activities and the liver microsomal hydroxylation system is a multicomponents system consisting of cytochrome P-450, NADPH-cytochrome c reductase and phosphatidylcholine (1,2). Recently, cytochrome P-450 has been purified from phenobarbital-treated or 3-methylcholanthrene-treated rats, rabbits and mice (3-7), and cytochromes P-450 and P-448 purified from these animals have been shown to be different hemoprotein one another based on their electrophoretic profiles on SDS-polyacrylamide gels and their catalytic, spectral and immunological properties (7). On the other hand, properties of guinea pig liver microsomal cytochrome P-450 have not yet been clear. In this communication, we would like to report on some properties of cytochrome P-450 isolated from phenobarbital-treated guinea pig liver microsomes.

MATERIALS AND METHODS

Materials - NADP, NADPH and cytochrome c (horse heart) were purchased from Boehringer Mannheim and dilauroyl-L-3-phosphatidylcholine was from Serdary Research Laboratories, Canada. Emulgen

0006-291X/80/0801020-07$01.00/0

913 was kindly supplied by Kao-Atlas Co., Japan. Sepharose 4B was obtained from Pharmacia Fine Chemicals and hydroxylapatite (Bio-Gel HT) was from Bio-Rad.

Male albino guinea pigs weighing 500 to 600 g were given 0.1 % sodium phenobarbital added to the drinking water for five days. The animals were fasted for 20 hr prior to sacrifice. Liver microsomes were prepared as described previously (8). Protein was determined by the method of Lowry et al. (9) using bovine serum albumin as a standard.

Purification of cytochrome P-450 and NADPH-cytochrome c reductase - Purification of cytochrome P-450 from phenobarbital-treated guinea pig liver microsomes was conducted using the ω-amino-n-octyl Sepharose 4B affinity column method of Imai et al (3,4).

Partial purification of NADPH-cytochrome c reductase from untreated guinea pig liver microsomes was conducted by minor modification of the method of Yasukochi and Masters (10). The partially purified reductase was free of cytochromes P-450 and b_5. Specific activity of the reductase was 34.2 μmole cytochrome c reduced per min.

Assay of cytochrome P-450 and NADPH-cytochrome c reductase activity - The concentration of cytochrome P-450 was determined by the method of Omura and Sato (11) from the CO-difference spectra of dithionite-reduced forms using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm. Cytochrome b_5 was determined by the measurement of dithionite-reduced minus oxidized difference spectra. All of these spectral measurements were carried out using an Aminco recording spectrophotometer, Model DW-2. For assay of the reductase activity the reduction of cytochrome c was measured according to the method of Phillips and Langdon (12).

Assay of hydroxylase activities - Benzphetamine N-demethylation by reconstituted system was determined by the rate of NADPH disappearance at 340 nm. Activities of benzopyrene hydroxylation and 7-ethoxycoumarin O-deethylation were measured fluorometrically according to the methods of Yang and Kicha (13) and Prough et al. (14), respectively.

RESULTS

Table I shows a summary of a typical purification experiment. About 50 to 60 per cent of cytochrome P-450 applied to a ω-amino-n-octyl Sepharose 4B column was eluted by washing the column with 0.1 M potassium phosphate (pH 7.25) containing 20 % glycerol, 0.4 % sodium cholate, 0.08 % Emulgen 913 and 1 mM dithiothreitol. The peak fractions of the eluate having an optical density at 417 nm greater than 1.0 were combined. The combined fraction was diluted 3-fold with 20 % glycerol and applied to a column of hydroxylapatite. The column was washed with 35 mM, 90 mM and 150 mM potassium phosphate (pH 7.25) all containing 20 % glycerol and 0.2 % Emulgen 913. This washing procedure eluted about 2 to 8 %

Table I Purification of cytochrome P-450 from liver microsomes of phenobarbital pretreated guinea pig

Fraction	Protein (mg)	Cytochrome P-450 T.C.* (nmoles)	Cytochrome P-450 S.C.** (nmoles/mg)	Recovery (%)
Microsomes	2145	4156	1.94	100
Solubilized supernatant	1659	4090	2.47	98
Aminooctyl eluate	215	2473	11.50	60
Hydroxylapatite eluate	67.0	858.6	12.81	21
CM-Sephadex eluate	26.8	381.8	14.25	9
Hydroxylapatite eluate	9.1	180.1	19.80	4

* Total content ** Specific content

of cytochrome P-450 applied but majority remained adsorbed to the column. The remained cytochrome P-450 was eluted by washing the column with 150 mM potassium phosphate (pH 7.25) containing 20 % glycerol, 0.2 % Emulgen 913 and 0.1 % sodium cholate. The peak fractions of eluate were combined and diluted 7.5-fold with 20 % glycerol containing 0.2 % Emulgen 913. The diluted solution was applied to a CM-Sephadex (C-50) and then the column was washed stepwise with 100 mM potassium phosphate (pH 7.25) and 150 mM potassium phosphate (pH 7.25) both containing 20 % glycerol and 0.2 % Emulgen 913. About 40 % of cytochrome P-450 bound was eluted with 100 mM potassium phosphate and only 3 to 4 % with 150 mM potassium phosphate; the later eluate had low specific contents. The main fractions of eluate were combined and diluted 3-fold with 20 % glycerol. The diluted solution was applied to a hydroxylapatite. After the column was washed with 35 mM potassium phosphate (pH 7.25) containing 20 % glycerol, cytochrome P-450 was eluted with 300 mM potassium phosphate (pH 7.25) containing 20 % glycerol.

The absorption spectra of purified cytochrome P-450 are shown in Fig. 1. The absolute absorption maximum of the purified cyto-

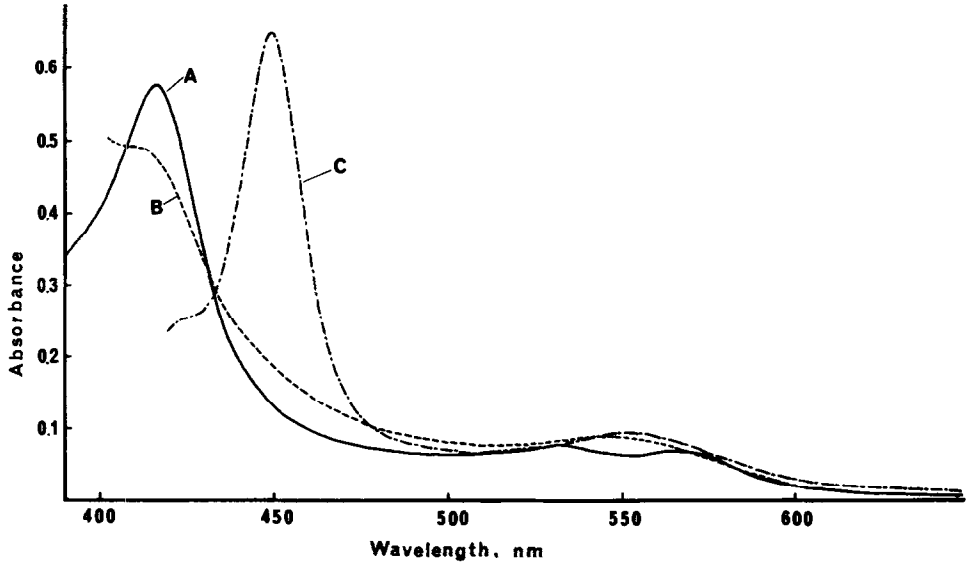

Fig. 1 Absorption spectra of purified cytochrome P-450 in 100 mM potassium phosphate, pH 7.25, containing 20 % glycerol. Curve A : oxidized form Curve B : dithionite-reduced form Curve C : carbon monoxide complex of reduced form.

chrome are as follows: oxidized 417, 532, 567 nm ; reduced 414, 544 nm ; CO-reduced 450, 552 nm. The fact that only a slight shoulder is seen at 423 nm in the spectrum of the CO compound indicates that the preparation was free of both cytochromes P-420 and b_5 (15).

When the purified preparation was subjected to polyacrylamide gel electrophoresis in the presence of 2 % sodium dodecylsulfate by the method of Laemmeli (16), a single protein band was observed, as shown in Fig. 2. When compared to proteins of known mobility, an apparent molecular weight of about 49,000 was estimated.

As shown in Table II, the purified preparation of cytochrome could catalyze NADPH-dependent N-demethylation of benzphetamine when mixed with an NADPH-cytochrome c reductase and dilauroyl-L-3-phosphatidylcholine as the source of phospholipid. As can be seen, cytochrome P-450 and NADPH-cytochrome c reductase were obligatorily

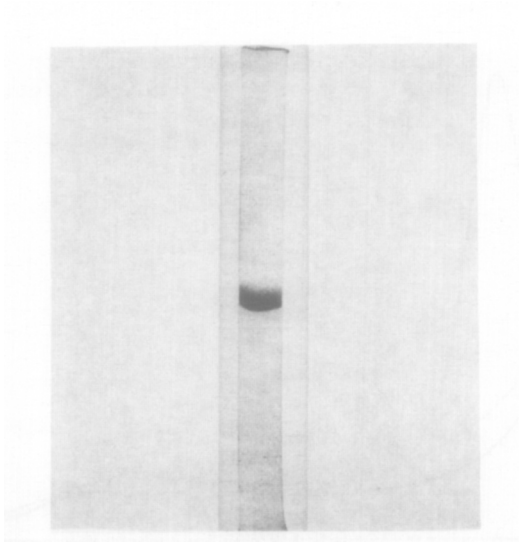

Fig. 2 SDS-polyacrylamide gel electrophoresis of purified cytochrome P-450. About 15 μg of protein was treated with 2 % SDS, 10 % glycerol and 5 % 2-mercaptoethanol and subjected to electrophoresis on a 7.5 % gel disc, as described by Laemmeli (16).

required for reconstitution of the N-demethylation of benzphetamine. The activity of various substrates in the reconstituted enzyme system containing cytochrome P-450 purified from phenobarbital-treated guinea pig or cytochrome P-448 partially purified from 3-methylcholanthrene-treated guinea pig, along with the reductase and dilauroyl-L-3-phosphatidylcholine, are presented in Table III.

Table II Reconstitution of benzphetamine N-demethylase activity

System	Benzphetamine N-demethylation (nmole NADPH oxidized/min/ml)	% Maximal activity
Complete	2.02	100
- P-450	0.13	6.4
- Reductase	0.15	7.2
- DLPC	0.87	43.0
- $MgCl_2$	1.28	63.3

The complete system contained (in a final volume of 1 ml) 0.1 M Na,K-phosphate buffer (pH 7.4), 0.05 nmole of cytochrome P-450, 0.2 unit of NADPH-cytochrome c (P-450) reductase, 30 μg of dilauroyl-L-3-phosphatidylcholine (DLPC), 0.1 mM EDTA, 10 mM $MgCl_2$ and 1 mM benzphetamine. The change in absorbance at 340 nm was measured using an Aminco recording spectrophotometer, Model DW-2.

Table III Mixed-function oxidase activities supported by purified cytochrome P-450 from phenobarbital-treated guinea pig in reconstituted system

Cytochrome	Benzphetamine* N-demethylation	Benzpyrene** hydroxylation	7-Ethoxycoumarin** O-deethylation
P-450	47.1	0.56	0.22
P-448***	9.6	2.56	5.96

* nmole NADPH oxidized per nmole cytochrome P-450 per min
** nmole product formed per nmole cytochrome P-450 per min
*** Cytochrome P-448 was partially purified from 3-methylcholanthrene-treated (25 mg/kg, 5 days, i.p.) guinea pig liver microsomes as described by Imai et al (3,4). Specific content of cytochrome P-448 used was 9.30 nmole per mg of protein.

Clearly, cytochrome P-450 is rather inactive in supporting benzopyrene hydroxylation and 7-ethoxycoumarin O-deethylation, and cytochrome P-448 is rather inactive in benzphetamine N-demethylation.

DISCUSSION

It has been shown that there are marked differences in catalytic activities of different forms of cytochrome P-450 and rabbit cytochrome P-448 is only about 3 per cent as active as rat cytochrome P-448 in supporting the hydroxylation of benzopyrene (7).

The cytochrome P-450 preparation cross reacts with antibody produced in rabbit against cytochrome P-450 purified from phenobarbital-treated rat liver microsomes (data not shown). As shown in Table III, benzphetamine N-demethylation was 5 times higher with cytochrome P-450 than with cytochrome P-448 and benzopyrene hydroxylation and 7-ethoxycoumarin O-deethylation were higher with cytochrome P-448 than with cytochrome P-450. From these results, it was suggested that in respect to catalytic and immunological properties, the purified cytochrome P-450 from phenobarbital-treated guinea pigs might be similar to that from phenobarbital-treated rats. However, further purification was needed to clarify the properties of cytochrome P-448 from guinea pig liver microsomes.

REFERENCES

1. Kato, R. (1979) Pharmacol. Therap. 6, 41-98.
2. Lu, A. Y. H. and Coon, M. J. (1968) J. biol. Chem. 243, 1331-1332.
3. Imai, Y. and Sato, R. (1974) Biochem. Biophys. Res. Commun. 60, 8-14.
4. Hashimoto, C. and Imai, Y. (1976) Biochem. Biophys. Res. Commun. 68, 821-827.
5. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., and Levin, W. (1976) J. biol. Chem. 251, 1385-1391.
6. Haugen, D. A. and Coon, M. J. (1976) J. biol. Chem. 251, 7929-7939.
7. Levin, W., Ryan, D., Huang, M.-T., Kawalek, J., Thomas, P. E., West, S. B. and Lu, A. Y. H. (1977) Microsomes and Drug Oxidations pl85-191 eds Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R. W. and Conney, A. H. Pergamon Press Oxford.
8. Kitada, M., Igarashi, T., Kamataki, T. and Kitagawa, H. (1977) Japan. J. Pharmacol. 27, 481-489.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. biol. Chem. 193, 265-275.
10. Yasukochi, Y. and Masters, B. S. S. (1976) J. biol. Chem. 251, 5337-5344.
11. Omura, T. and Sato, R. (1964) J. biol. Chem. 239, 2370-2378.
12. Phillips, A. H. and Langdon, R. G. (1962) J. biol. Chem. 237, 2652-2660.
13. Yang, C. S. and Kicha, L. P. (1978) Anal. Biochem. 84, 154-163.
14. Prough, R. A., Burke, M. D. and Mayer, R. T. (1978) Methods in Enzymology, 52, 372-377.
15. Imai, Y. and Sato, R. (1974) J. Biochem., Tokyo, 75, 689-697.
16. Laemmeli, U. K. (1970) Nature, 227, 680-685.