Vol. 81, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1978 Pages 19-23

ISOLATION AND PARTIAL PURIFICATION OF CYTOCHROME P-450 FROM UNINDUCED RAT LIVER

G. Hossein Dialameh

Department of Biochemistry
Faculty of Basic Medical Sciences
University of Tehran, Iran

Received November 28,1977

SUMMARY

Cytochrome P-450 has been partially purified from uninduced rat liver to a specific content of 9.6 nmoles/mg protein, and contains no cytochrome b_5 or NADPH-cytochrome c reductase. Microsomes were solubilized by Emalgen 913 followed by ammonium sulfate precipitation and column chromatography on DEAE-cellulose and calcium phosphate gel. The cytochrome P-450 is catalytically active when reconstituted with lipid and NADPH-cytochrome c reductase for hydroxylation of benzo[a]pyrene.

INTRODUCTION

Cytochrome P-450 has recently been purified from Pseudomonas putida (1), rabbit liver (2), and rat liver (3); and partial purification has been reported in mice (4). Most purification procedures employ induction of cytochrome P-450 with phenobarbital or 3-methylcholanthrene (2-4). Induction enlarges the liver, increases the concentration of cytochrome P-450 (5), and changes the nature of the cytochrome present (3,6,7). Because of these drug induced changes purification and reconstitution of cytochrome P-450 from uninduced animals would be informative about the state of cytochrome P-450 in normal animals.

Our interest in this work originated from our recent study of the hydroxylation of heterocyclic and polycyclic carcinogens by this system. however, this presents problems, larger amounts of material must be used to produce a suitable yield and the ratio of cytochrome P-450 to other microsomal proteins is very low. This paper reports our preliminary results on the development of a high yield procedure for the purification of cytochrome P-450, with catalytic activity from uninduced rat liver.

MATERIAL AND METHODS

Isolation of Microsomes: Two-month old male rats (Pasteur Institute, Tehran) were used for all experiments. Microsomes were prepared by the method

0006-291X/78/0811-0019$01,00/0

of Dialameh et al. (8) with some modifications. The rats were killed by decapitation and the livers were exised, washed 2 times in ice cold 1.15% KCl. Liver microsomes were prepared from 33% homogenates in 0.05 M Tris-acetate buffer (pH 7.5) containing 1.15% KCl, 10 mM EDTA. The final microsomal pellets were resuspended in 0.25 M sucrose at a protein concentration of 30-40 mg/ml and stored under nitrogen at -20° for 2-3 days prior to use.

Solubilization of Microsomal Protein: A microsomal suspension containing 2,150 mg of protein in 60 ml of 0.25 M sucrose was diluted to 150 ml with 20 ml of 1 M potassium phosphate buffer (pH 7.5), 30 ml glycerol, 2 ml of 0.1 M EDTA, and 0.25 M sucrose and sonicated in an ice salt bath (temperature below 8°) with a Biosonic III at full output for four 30-s intervals. Emalgen 913 (a polyoxyethylene nonylphenyl ether, Kao-Atlas Co. Ltd., Tokyo), 2 mg/mg protein was added. The mixture stirred for 20 min at 4° under nitrogen. The solubilized microsomal protein was centrifuged at 105,000 g for 60 min. The suppernatant was fractionated with ammonium sulfate. The 40 to 50% ammonium sulfate precipitate was dissolved in buffer, centrifuged at 105,000 g for 90 min and the red supernatant was dialyzed overnight. The per cent saturation of ammonium sulfate was calculated in the usual manner (9) despite the presence of glycerol in the solution. The dialyzed sample containing cytochrome P-450 was dissolved in 0.01 M potassium phosphate buffer (pH 7.7) containing 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA and 0.5% Emalgen 913 and was applied to a DEAE-cellulose column (2.8 X 48 cm) previously equilibrated with 0.005 M potassium phosphate buffer (pH 7.7) containing 0.1% Emalgen 913 and 20% glycerol. The proteins were eluted with a linear gradient of KCl (0 to 0.5 M) containing 0.005 M potassium phosphate buffer (pH 7.7), 0.1% Emalgen 913 and 20% glycerol. The fractions with high specific contents of cytochrome P-450 were pooled, concentrated by adsorbtion on calcium phosphate gel (4 mg of gel per mg of protein), and eluted from the gel 0.25 M potassium phosphate buffer (pH 7.7).

Assays of Cytochrome P-450: The concentration of cytochrome P-450 was determined by the method of Omura and Sato (10) from the CO-difference spectra of dithinite-reduced samples using an extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm. Cytochrome b_5 was measured by the method of Rogers and Strittamatter (11) using an extinction coefficient of 119 mM^{-1} cm^{-1}. All of the above spectra were recorded on a Cary model 118C spectrophotometer.

Partial Purification of NADPH-Cytochrome c Reductase: The reductase was partially purified from uninduced rat liver microsomes by the method of van der Hoeven and Coon (12). It was free of cytochrome P-450 and cytochrome b_5. For assay of the enzyme the reduction of cytochrome c was followed at 550 nm at 25° using an extinction coefficient of 21 mM^{-1} cm^{-1}. One unit of reductase is defined as nmoles of cytochrome c reduced per min. Lipids were extracted from microsomes by the method of Bligh and Dyer (13) as described by Holtzman and Gillete (14).

RESULTS

Table I showes a summary of a typical purification experiment. This procedure reproducibly yielded cytochrome P-450 having specific content of 8-11 nmoles per mg protein with over all yield of 28 %. The purified preparation of cytochrome P-450 (oxidized form) was stable in the presence of 20% glycerol and could be stored at least two weeks at -20° without any spectral changes. During longer standing and reconstitution study, cytochrome P-450 underwent slow conversion into the inactive cytochrome P-420 form even in the presence of glycerol. With proper control, the reconstituted cytochrome P-420 reconverted to active cytochrome P-540 using the method of Yu and Gunsalus (16) with some modifications.

Table I. Purification of cytochrome P-450 from liver microsomes of uninduced rats. The procedure is described in text. Protein was determined by the method of Lowry et al. (15).

Fraction	Protein	Cytochrome P-450		
	(mg)	T.C.* (nmole)	S.C.** (nmole/mg)	Recovery (%)
Microsomes	2,150	1,419	0.66	100
Solubilized supernatant	1,698	1,375	0.81	97
40-50% $(NH_4)_2SO_4$	969	1,163	1.20	82
DEAE-cellulose	64	497	7.80	35
Calcium phosphate gel	42	397	9.60	28

*Total content. **Specific content

Figure 1 showes the absorbtion spectra of purified cytochrome P-450. The absolute absorbtion maximum (nm) of the purified preparations are as follows: oxidized, 416, 537, 569; reduced, 418, 557; CO reduced, 450, 556. 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 b_5 and P-420 (17).

As shown in Table II, the purified preparation of cytochrome P-450 could catalyze NADPH-dependent hydroxylation of benzo[a]pyrene when mixed with an NADPH-cytochrome c reductase preparation and purified microsomal lipid. As can be seen (Table II), cytochrome P-450, the reductase, and NADPH were obligatorily required for reconstitution of the hydroxylation of benzo-[a]pyrene.

DISCUSSION

Imai and Sato (19) and van der Hoeven et al. (20) have independently reported a 6 fold purification of induced rabbit liver cytochrome P-450 to a specific content of about 17 nmoles/mg protein (compared with 3 nmoles P-450/mg of induced intact microsomes). Ryan et al. (21) reported a 10 fold purification of induced rat liver cytochrome P-450 to a specific content of about 17 nmoles/mg protein (compared with 1.7 nmoles P-450/mg of induced intact microsomes). In our procedure we have increased the specific content of cytochrome P-450 from uninduced liver by 14 fold to a level of about 9.6

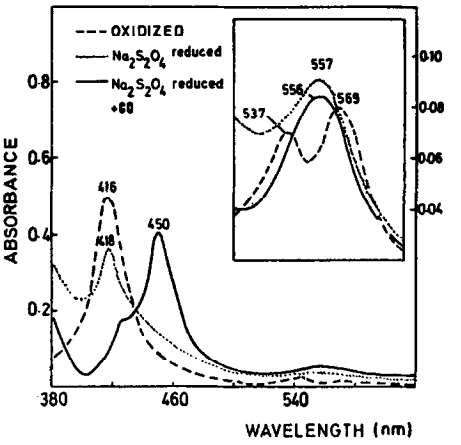

Figure 1. Absorbtion spectra of partially purified cytochrome P-450 containing 9.6 nmoles per mg of protein. It was diluted to a concentration of 3.0 nmoles per ml in 0.05 M Tris-acetate buffer (pH 7.4), containing 20% glycerol and 1 mM EDTA. Inset, spectrum at higher wavelengths with 10 fold expanded absorbance scale.

Table II. Reconstitution of benzo a pyrene hydroxylase activity. The full system contained (in final volume of 2 ml) 50 mM Tris-acetate buffer, pH 7.5, 1.2 nmole cytochrome P-450, 12.5 units of NADPH-cytochrome c reductase, 0.1 mg of lipid, 1 mM NADPH, 2 mM $MgCl_2$, and 10 mM benzo a pyrene. The mixture was incubated at 37° for 20 min. The hydroxylation of benzo[a]pyrene was determined by the method of Depierre et al. (16).

Experimental condition	Benzo[a]pyrene hydroxylase activity (nmoles/mg protein)	
	Expt 1	Expt 2
Full system	0.168	0.161
- P-450	0.010	0.015
- Reductase	0.020	0.020
- NADPH	0.005	0.002
- Lipid	0.070	0.064

nmoles/mg protein (Table I). The cytochrome P-450 is catalytically active when reconstituted with lipid and NADPH-cytochrome c reductase. The full system also catalyzed hydroxylation of testosterone and progesterone (data not shown). The reconstituted benzo a pyrene hyroxylase activity was about one third that of starting liver microsomes on the basis of cytochrome P-450

content (Table II). However, it is expected that this activity will be further improved by refinement of reconstitution conditions.

The results of these experiments indicate that a method developed along the lines reported here will enable us to purify cytochrome P-450 with catalytic activity from uninduced rat liver with high recovery. Further experiments to study the catalytic properties of the partially purified cytochrome P-450 reported in this paper for the hydroxylation of several heterocyclic and polycyclic carcinogens are now in progress in our laboratory.

REFERENCES

1. Yu, C-A, Gunsalus, I. C., Katagiri, M., Suhara, K., and Takemori, S. (1974) J. Biol. Chem. 249, 94-101.
2. Haugen, D. A., and Coon, M. J. (1976) J. Biol. Chem. 251, 7929-7939.
3. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., and Levin, W. (1976) J. Biol. Chem. 251, 1385-1391.
4. Nebert, D., Heidema, J. K., Trobel, H. W., and Coon, M. J. (1973) J. Biol. Chem. 248, 7631-7636.
5. Conney, A. H. (1967) Pharmacol. Rev. 19, 316-366.
6. Sladek, N. E., and Mannering, G. J. (1966) Biochem. Biophys. Res. Comm. 24, 668-674.
7. Comai, K., and Gaylor, J. L. (1973) J. Biol Chem. 248, 4946-4955.
8. Dialameh, G. H., Yekundi, K. G., and Olson, R. E. (1970) Biochim. Biophys. Acta 223, 332-338.
9. Green, A. A., and Hughes, W. L. (1955) Methods Enzymol. 1, 76.
10. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
11. Rogers, M. J., and Strittamatter, P. (1973) J. Biol. Chem. 249, 895-900.
12. van der Hoeven, T. A., and Coon, M. J. (1974) J. Biol. Chem. 249, 6302-6310.
13. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
14. Holtzman, J. L., and Gillette, J. R. (1968) J. Biol. Chem. 193, 265-275.
15. Lowry, O. H., Rosenbrough, N.J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
16. Yu, C-A, and Gunsalus, I. C. (1974) J. Biol. Chem. 249, 102-106.
17. Imai, Y., and Sato, R. (1974) J. Biochem. 75, 689-697.
18. DePierre, J. W., Moron, M. S., Johannesen, K. A. M., and Ernster, L. (1975) Anal. Biochem. 63, 470-484.
19. Imai, Y., and Sato. R. (1974) Biochem. Biophys. Res. Comm. 60, 470-484.
20. van der Hoeven, T. A., Haugen, D. A., and Coon, M. J. (1974) Biochem. Biophys. Res. Comm. 60, 569-575.
21. Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B., and Levin, W. (1975) Biochem. Biophys. Res. Comm. 164, 1134-1141.