Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 826-832

PARTIAL PURIFICATION OF HUMAN LIVER CYTOCHROME P 450

Ph. BEAUNE[X], P. DANSETTE[XX], J.P. FLINOIS[X], S. COLUMELLI[X]
D. MANSUY[XX] and J.P. LEROUX[X]

[X] Laboratoire de Biochimie, U 75 INSERM, Faculté de Médecine Necker-Enfants Malades, 156. rue de Vaugirard 75730 Paris Cédex 15

[XX] Laboratoire de Chimie de l'Ecole Normale Supérieure, Laboratoire associé du CNRS n° 32, 24. rue Lhomond 75231 Paris Cédex 05, France

Received March 20,1979

SUMMARY

Human liver cytochrome P 450 was partially purified by hydrophobic chromatography on Octyl-Sepharose, followed by ion-exchange chromatography on DEAE-cellulose. Two fractions (A and B) were obtained ; cytochrome P 450 of fraction A was purified sixfold, with an overall yield of about 6 %. Its spectral properties were similar to those previously described in animal cytochromes P 450. Moreover, p-nitroanisole-O-demethylase activity could be obtained in a reconstituted system involving cytochrome P 450 of fraction A, human NADPH-cytochrome c reductase and phospholipids.

INTRODUCTION

Cytochromes P 450 from various animal species have been largely studied and purified (1-10), but very few results have been as yet obtained in man (11-14). However, it seems of prominent pharmacotoxicological interest to separate and study the different forms of human liver cytochrome P 450, compare them to known animal forms, and determine their relative specificities towards various substrates.

Having the opportunity to get fresh human liver from kidney transplantation donors, we have used for human microsomes a purification method that we had previously developed for rat cytochromes P 450, and which mainly consists in an hydrophobic chromatography on Octyl-Sepharose (14) followed by classical DEAE-(9) and CM-cellulose (5) chromatography. Preliminary results concerning a partial purification of human liver cytochrome P 450 are presented here.

MATERIAL AND METHODS

Purification procedure : Human livers were obtained from kidney transplantation donors. Liver was removed within 1 hour after circulatory arrest, immediately stored in solid CO_2, then at - 80°C. In the example shown here, the donor was a 40-years-old man dead of cranial traumatism. 100 g of frozen liver were cut into small pieces, then homogenized in 500 ml of 250 mM su-

0006-291X/79/110826-07$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

crose, 1 mM Tris-HCl, 1 mM EDTA, pH 7.4, successively with a Sorvall omnimix and a Potter apparatus. Microsomes were prepared as previously described (15), and solubilized in buffer A : 10 mM phosphate pH 7.4, 1 mM EDTA, 500 mM NaCl, 20 % (v/v) glycerol, 0.5 % (w/v) sodium cholate (Sigma) ; cytochrome P 450 concentration was adjusted to about 1 nmol per ml of buffer A (1.6 mg cholate x mg^{-1} microsomal proteins) ; the microsomal suspension was kept for 30 min under gentle agitation then centrifuged for 30 min at 100 000 g. The supernatant was applied to an Octyl-Sepharose CL-4B (Pharmacia Chemicals) column, previously equilibrated, then washed with buffer A. Elution was performed with buffer B : 10 mM phosphate pH 7.4, 0.1 mM EDTA, 20 % glycerol, 0.5 % sodium cholate, 0.2 % Emulgen 911 (Kao Atlas). After elution of the first cytochrome b_5 fraction, buffer C (similar to buffer B, but containing 0.5 % Emulgen) was used. Absorption was continuously monitored at 417 nm with a Holochrom spectrophotometer (Gilson medical electronics). Cytochrome P 450-containing fractions were pooled, concentrated by ultrafiltration on a PM 10 Amicon Membrane, and applied, as described by Warner et al. (9), to a DEAE-cellulose (DE 52 Whatman) column previously equilibrated, then washed with buffer B, and eluted by a linear gradient (0 to 250 mM NaCl in buffer B), followed by two steps at 250 and 500 mM NaCl in buffer B. Two peaks of cytochrome P 450 were generally obtained. In one case, these fractions were concentrated by ultrafiltration, dialyzed for 6 hours against buffer D (5 mM phosphate pH 6.5, 0.1 mM EDTA, 20 % glycerol, 0.05 % Emulgen 911) and applied to a CM-cellulose (CM 52 Whatman) (5) column previously equilibrated then washed with buffer D. Elution was performed with buffer D containing 100 mM phosphate. Final preparations of cytochrome P 450 were neutralized to pH 7.4 if necessary, concentrated by ultrafiltration, treated by Biobeads SM 2 (Biorad Laboratories), precipitated by 25 % polyethylene glycol 6000 (Baker), and centrifuged for 30 min at 100 000 g. Pellet was dissolved in 100 mM phosphate pH 7.4, containing 20 % glycerol, and stored at - 80°C. All purification steps were performed at 4°C.

Reconstitution of enzymatic activity : Phospholipids were extracted from 5 ml of human microsomes (30 mg of proteins/ml) by 3 x 15 ml chloroform methanol (2/1, v/v), then purified as described (16). They were suspended in 50 mM Tris-HCl pH 7.4, containing 150 mM KCl and 10 mM $MgCl_2$, and sonicated before use. Reconstitution experiments were performed as follows (p-nitroanisole-O-demethylase activity) : 1 ml of medium contained 100 µmoles of phosphate (pH 7.4), 200 mg of glycerol, human NADPH-cytochrome c reductase (first non adsorbed peak on Octyl-Sepharose column, fraction a of fig. 1a, 200 nmol of cytochrome c reduced x min^{-1}), 200 µg of cholate, 40 µg of human microsomal phospholipids, 1.5 µmoles of p-nitroanisole, and 0.3 to 1.2 nmoles of cytochrome P 450 ; this mixture was preincubated at 37°C for 20 min, and assay was initiated by adding the NADPH-generating system (5 µmoles of glucose-6-phosphate, 0.3 µmoles of NADP and 2 U of glucose-6-phosphate dehydrogenase) ; enzymes and substrates were purchased from Boehringer Mannheim.

Assays : Proteins (17), cytochrome P 450 (18), NADPH-cytochrome c reductase (19) and p-nitroanisole-O-demethylase (20) were determined according to described methods. Spectra were recorded on an Aminco DW 2 or Beckman Acta M 6 spectrophotometer. Electrophoresis were performed as described by Laemmli (21) on polyacrylamide gels (15 cm length) in the presence of sodium dodecylsulfate.

RESULTS

Fig. 1 shows the elution profile of an Octyl-Sepharose column loaded with human liver microsomes after cholate solubilization. The first peak (a) corresponding to an absorption at 417 nm contained NADPH-cytochrome c reductase and hemoglobin, but no cytochrome P 450. After ultrafil-

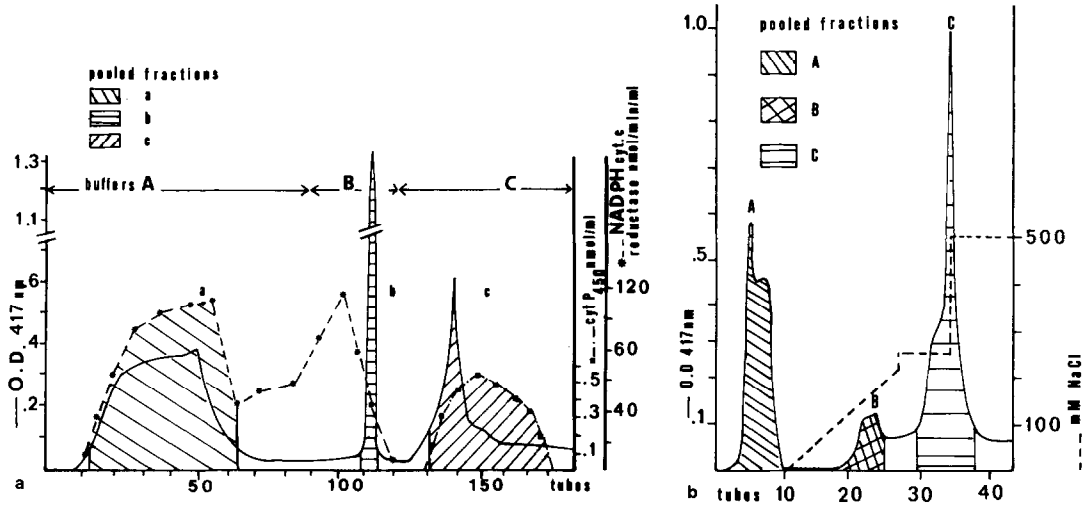

Figure 1a : Elution profile of human solubilized microsomes on an Octyl-Sepharose 4 B column.

335 nmoles of cytochrome P 450, solubilized in buffer A, were applied to the column (2,5 cm internal diameter, 30 cm height), and elution was performed by buffers A, B and C as described in the text. Fraction a : NADH-cytochrome c reductase and hemoglobin ; fraction b : cytochrome b_5 ; fraction c : cytochrome b_5 and cytochrome P 450.

Figure 1b : Elution profile of cytochrome P 450 and b_5 (fraction c of Octyl-Sepharose eluate) on a DEAE-cellulose column.

72 nmoles of cytochrome P 450 were applied to the column (1,5 cm internal diameter, 30 cm height) and eluted as described in the text. Fractions A and B : cytochromes P 450 ; fraction C : cytochrome b_5.

tration and dialysis, it was used in the reconstitution system (table I). Another reductase peak, devoid of hemoglobin, could also be used in the reconstitution system with the same results. The second 417 nm-absorption peak (b) was mainly constituted by cytochrome b_5, whereas the third peak (c) contained a mixture of cytochrome b_5 and cytochrome P 450. After ultrafiltration, peak c was applied to a DEAE-cellulose column which allowed the separation of two cytochrome P 450 fractions (A and B) and one cytochrome b_5 fraction. The small B fraction was lost during polyethylene glycol precipitations. Fraction A, treated as described under material and methods, was used for spectral and reconstitution studies. Table I summarizes the whole purification procedure : fraction A was purified about sixfold with a final yield of about 6 %. It did not contain cytochrome b_5, and its NADPH-cytochrome c reductase content was very low (1.3 nmol cytochrome c reduced x

Table I - SUMMARY OF A PURIFICATION OF HUMAN CYTOCHROME P 450 AND RECONSTITUTION OF CATALYTIC ACTIVITY

	Cytochrome P 450			
	Total Content (nmoles)	yield %	Specific activities (nmol x mg^{-1} protein)	Purification (fold)
Microsomes	491	100	0.54	1
Solubilized microsomes	334	68	0.40	0.7
Octyl-Sepharose eluate (fraction c)	79	16	1.7	3.1
DEAE-cellulose eluate (fraction A)	30	6.1	3.2	5.9

	Reconstituted p-nitroanisole-O-demethylase activity of fraction A (nmol p-nitrophenol produced x min^{-1} x $nmol^{-1}$ cytochrome P 450)
Complete system	0.55 $\pm$ 0.08 (3 determinations)
Complete system in the presence of 1 mM SKF 525 A or CO	0 (2 determinations)
Fraction A heated for 5 min a 95°C	0 (1 determination)

See text for experimental details

min^{-1} per nmol of cytochrome P 450). By polyacrylamide SDS electrophoresis, fraction A yielded a major band with a molecular weight of about 45 000. As shown on fig. 2, the absolute spectrum of oxidized cytochrome P 450, as well as its absolute and difference spectra with a pyridine derivative, were very similar to those previously described in animal cytochromes. Cytochrome P 450-Fe(II)-CO absolute spectrum exhibited a Soret peak at 450 nm, but also a second minor peak at 420 nm. p-nitroanisole-O-demethylase activity could be obtained with a reconstituted system involving cytochrome P 450 of fraction A, NADPH-cytochrome c reductase and phospholipids (table I). This activity increased linearly with cytochrome P 450 concentration (three concentrations were tested), and disappeared completely by heating fraction A, or adding CO or SKF 525 A. It was not possible to reconstitute 7-ethoxycoumarin-O-deethylase activity under the same experimental conditions.

DISCUSSION

Hydrophobic chromatography on Octyl-Sepharose was previously developed in our laboratory for the separation of rat liver cytochromes P 450, leading to well purified fractions which, in the best cases, contained 18

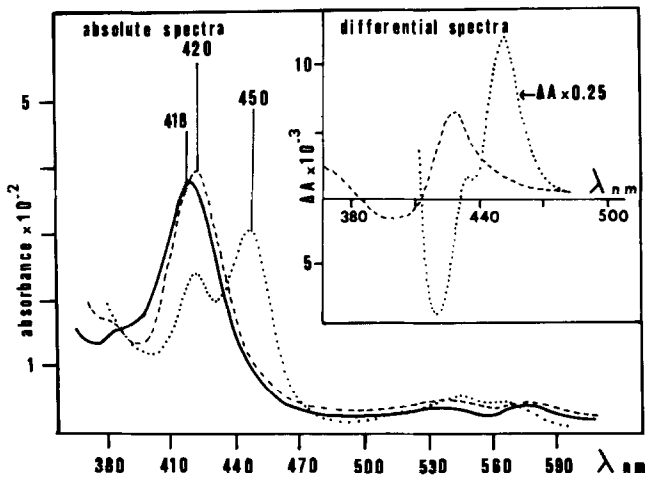

Figure 2 : Spectral characteristics of human cytochrome P 450 (fraction A)
——— Cyt P 450-Fe(III) ; — — — — cyt P 450-Fe(III) + 1 mM ethylpyridine ; ····· cyt P 450-Fe(II)-CO. In absolute spectra, reference cuvette contained 20 % (v/v) glycerol in a 100 mM phosphate buffer pH 7.4 and sample cuvette 0.38 µM cytochrome P 450 in the same buffer. In differential spectra the two cuvettes contained 0.38 µM cytochrome P 450 in the phosphate buffer. Ethylpyridine was added in the sample cuvette. For CO spectra, CO was bubbled in the sample cuvette after addition of sodium dithionite in sample and reference cuvettes.

nmol of cytochrome P 450 per mg protein (14). This method is particularly interesting in the case of human microsomes, heavily contaminated by hemoglobin, since it allows a rapid elimination of this hemoprotein which is not retained. Moreover, two NADPH-cytochrome c reductase fractions are obtained ; they are devoid of Emulgen, cytochrome b_5 and cytochrome P 450, and may be used in reconstitution experiments after concentration by ultrafiltration. Finally, the cytochrome P 450 fraction may be, after ultrafiltration, directly applied to the DEAE-cellulose column which gives elution profiles quite similar to those obtained in non-induced rat (high peak A, low peak B). Cytochrome b_5 is eluted at much higher NaCl concentrations (250 to 500 mM). This simple method rapidly leads to a sixfold purified cytochrome P 450 fraction, with an overall yield of about 6 %. It also yields fractions enriched in cytochrome b_5 and NADPH-cytochrome c reductase which will be further purified and studied. The cytochromes P 450 of fractions A and B may be further fractionated on CM-cellulose (work presently in progress) : in one experiment, A could be separated into two fractions and B into three ; as yet, the best purification of cytochrome P 450 was obtained in fraction A_2 (8 nmol cytochrome P 450 x mg^{-1} protein).

The spectral properties of partially purified human cytochrome P 450 (fraction A) in its oxidized form are quite similar to those of purified rat or mouse cytochromes P 450 (5, 6, 14) ; this cytochrome is also able to bind pyridine compounds, leading to a slight red shift of the Soret peak of the absolute spectrum, and a maximum at 428 nm in the difference spectrum (type II). Moreover, the absolute spectrum of human cytochrome P 450-Fe(II)-CO exhibits a Soret peak at 450 nm. The presence of a 420 nm peak in this system may be due to a denaturation of cytochrome P 450 by excess dithionite which seems necessary for a sufficiently fast reduction of human purified cytochrome P 450.

The partially purified human cytochrome P 450 has a molecular weight of about 45 000 and exhibits p-nitroanisole-O-demethylase reconstituted activity. The observed molecular activity is lower than usually observed in human microsomes (about 3 nmol x min^{-1} x $nmol^{-1}$ cytochrome P 450). This lower activity could be due to (i) imperfect reconstitution conditions, (ii) a partial loss of the catalytic properties of cytochrome P 450 during purification, or (iii) a low p-nitroanisole-O-demethylase activity of the cytochrome P 450 forms of fraction A. The study of the monooxygenase activities of fraction B, presently in progress, will allow a comparison with human fraction A, and homologous rat fractions.

ACKNOWLEDGEMENTS

The authors wish to thank Paris-Transplant, Dr Frantz and Dr Kreiss for providing fresh liver samples. This work was supported by grants from DGRST (Biologie de la Reproduction et du Développement) and CNRS (ATP A 651-3763).

REFERENCES

1. Gibson, G.G. and Schenkman, J.B. (1978) J. Biol. Chem. 253, 5957-5963.
2. Guengerich, F.P. (1977) J. Biol. Chem. 252, 3970-3979.
3. Hashimoto, C. and Imai, Y. (1976) Biochem. Biophys. Res. Commun. 68, 821-827.
4. Haugen, D.A. and Coon, M.J. (1976) J. Biol. Chem. 251, 7929-7939.
5. Huang, M., West, S.B., Lu, A.Y.H. (1976) J. Biol. Chem. 251, 4659-4665.
6. Imai, Y. and Sato, R. (1974) Biochem. Biophys. Res. Commun. 60, 8-14.
7. Philpot, R.M. and Arinc, E. (1976) Mol. Pharmacol. 12, 483-493.
8. Thomas, P.E., Lu, A.Y.H., Ryan, D., West, S.B., Kawalek, J. and Levin, W. (1976) Mol. Pharmacol. 12, 746-758.
9. Warner, M., Lamarca, M.V. and Neims, A.H. (1978) Drug Metab. Dispos. 6, 353-362.
10. Yu, C.A., Gunsalus, I.C., Katagiri, M., Suhara, K. and Takemori, S. (1974) J. Biol. Chem. 249, 94-101.
11. Bjorkhem, I., Kager, L., Wikvall, K. (1976) Biochem. Med. 15, 87-94.
12. Kamataki, T., Yamazoe, Y., Sugiura, M. and Kato, R. (1978) Jpn. J. Pharmacol. 28 (S), 155.
13. Kaschnitz, R.M. and Coon, M.J. (1975) Biochem. Pharmacol. 24, 295-297.

14. Dansette, P., Beaune, Ph., Flinois, J.P., Lostanlen, D., Mansuy, D. and Leroux, J.P. (1978), 7th congress of pharmacology, satellite symposium on "isolated drug metabolizing enzymes", Mainz.
15. Degwitz, E., Ullrich, V., Staudinger, H. and Rummel, W. (1969) Hoppe Seyler's Z. Physiol. Chem. 350, 547-553.
16. Artom, C. in Methods in enzymology, Colowick, S.P., Kaplan, N.O. (Eds) (1957), 4, 823, Academic Press, New York.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
18. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370.
19. Mazel, P. in Ladu, B.N., Mandel, H.G. and Way. E.L. (Eds)(1971) Fundamentals of drug metabolism and drug disposition. Chap. 27, p. 546, Williams and Wilkins, Baltimore.
20. Zannoni, V.G., in Ladu, B.N., Mandel, H.G. and Way. E.L. (Eds) (1971) Fundamentals of drug metabolism and drug disposition. Chap. 27, p. 556, Williams and Wilkins, Baltimore.
21. Laemmli, U.K. (1970) Nature, 227, 680-685.