A Simple and Rapid Method for the Purification of Cytochrome P-450 (Form LM4)

Michael A. Alterman* **and Andrew I. Dowgii**
Department of Biochemistry, 2nd Moscow Medical Institute, 117869 Ostrovitjanova 1, Moscow, USSR

A one step chromatographic method for the purification of membrane protein cytochrome P-450 LM4 has been developed and it indicates that CM Sepharose Fast-Flow is an excellent material for cytochrome P-450 LM4 chromatography.

INTRODUCTION

Cytocrome P-450 is a group of membrane bound monooxygenases located in the microsomal fraction from many tissues (Nebert *et al.*, 1987). As a rule, cytochrome P-450's purification includes, at least three chromatographic steps, starting with affinity chromatography of the detergent-treated microsomes on an aminooctyl-Sepharose column followed by ion-exchange chromatography and ending with hydroxyapatite chromatography (Tamburini *et al.*, 1984 and Guengerich *et al.*, 1982). We have found that cytochrome P-450 (form LM4), from liver microsomes of male rabbits treated with 3-methylcholanthrene (3-MC), can be purified rapidly and effectively in one step.

EXPERIMENTAL

Materials and chemicals. DEAE-Sephacel, DEAE-Sepharose, and CM-Sepharose Fast-Flow were purchased from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from either Sigma Chemical Co. (St Louis, MO, USA) or Serva Feinbiochemica Co. (Heidelberg, West Germany).

Animals and drug pretreatment. Male rabbits (7–9 weeks old) had been pretreated with 3-MC (80 mg/kg i. p. for 3 days) and killed 24 h after the last dose.

Preparation of rabbit liver microsomes. 3-MC microsomes were prepared using conventional ultracentrifugation as described elsewhere (Devichenskii *et al.*, 1979).

Purification of rabbit liver cytochrome P-450 LM4. 3-MC microsomes were solubilized in 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.625% sodium cholate and 1.25% Triton N-101 (cf. procedure for the purification of NADPH-cytochrome P-450 reductase, Yasukochi and Masters (1976). The solution obtained was applied to a DEAE-Sephacel (or DEAE-Sepharose) column (5 × 30 cm). The uncoupled material (eluate) was applied to the CM Sepharose Fast-Flow column (1.6 × 40 cm), preequilibrated with 20 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 20% glycerol. When loaded, a dark brown band formed at the top of the column. The loaded column was then washed with 50 mM potassium phosphate buffer pH 7.4 containing, 1 mM DTT and 20% glycerol, followed by 100 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 20% glycerol. Cytochrome was then eluted with 300 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 20% glycerol. All procedures were carried out at 4 °C.

Separation of cytochrome P-450 LM4 from cytochrome P-420. The cytochrome P-450 LM4 solution was diluted 15-fold with 20 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 0.2% emulgen 913. It was then applied to CM Sepharose Fast-Fow column (1 × 30 cm), preequilibrated with the same buffer. The column was washed with 50 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 0.2% emulgen 913 and the final elution was carried out with 150 mM potassium phosphate buffer pH 7.4 containing 1 mM DTT and 0.2% emulgen 913. The partially inactivated cytochrome was eluted first.

PAGE-SDS. This was performed according to Laemmli (1970) using a linear acrylamide gradient 8–15%. A mixture of proteins from Pharmacia Fine Chemicals (Uppsala, Sweden) was used as molecular weight markers.

Sequence determinations. Manual sequence analysis of aliquots of the proteins were performed by the combined use of 4-*N*,*N*′-dimethylaminoazobenzene-4′-isothiocyanate and phenylisothiocyanate (Wittmann-Liebold *et al.*, 1986). Identification of dimethylaminoazobenzene thiohydantoin amino acids was carried out by thin layer chromatography on 2.5 × 2.5 cm polyamide sheets (Schleicher and Schull, Dassel, West Germany).

Analytical method. The protein concentration was determined by the method of Lowry *et al.* (1951). The concentration of cytochrome P-450 was calculated according to Omura and Sato (1967) from CO difference spectra.

Table 1. A typical purification of cytochrome P-450 LM4 from liver microsomes of 3-MC treated rabbits. The purification was carried out as described in Experimental.

Preparation	Total content of cytochrome P-450 LM4 (nmol)	Recovery %
Solubilized microsomes	3200	100
DEAE-Sephacel	1600	50
CM Sepharose Fast-Flow	1014	32

* Author to whom correspondence should be addressed.

0269-3879/90/0221-0222 $5.00

RESULTS AND DISCUSSION

The present method for the purification of cytochrome P-450 LM4 combines a very high yield with simplicity (up to 30%, see Table 1, cf. Tamburini *et al.*, 1984, Guengerich *et al.*, 1982, Hashimoto and Imai 1976). Moreover it is possible to apply this one-step procedure for the simultaneous purification of cytochrome P-450 LM4 and NADPH-cytochrome P-450 reductase. N-Terminal analysis of the electrophoretically homogeneous cytochrome P-450 LM4 preparation reveals the sequence Ala-Met-Ser-Pro-Ala (cf. Ozols, 1986; Okino *et al.*, 1985).

The method was based on our initial observation that it was possible to achieve chromatographic separation of cytochrome P-450 LM4 from its inactivated form (so-called cytochrome P-420) on the CM Sepharose Fast-Flow column. We therefore conclude that CM Sepharose Fast-Flow is an ideal material for the separation of cytochrome P-450 LM4.

REFERENCES

Devichenskii, V. M., Alterman, M. A., Zikhareva, V. O. *et al* (1979). *Biokhimia* **44**, 748.

Guengerich, F. P., Dannan, G. A., Wright, S. T. and Martin, M. V. (1982). *Xenobiotica* **12**, 701.

Hashimoto, C. and Imai, Y. (1976). *Biochem. Biophys. Res. Commun.* **68**, 821.

Laemmli, U. K. (1970). *Nature* **227**, 681.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall R. J. (1951). *J. Biol. Chem.* **193**, 265.

Nebert, D. W., Adesnik, M., Coon, M. J. *et al* (1987). *DNA* **6**, 1.

Okino, S. T., Quatrochi, L. C., Barnes, H. J. *et al* (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5310.

Omura, T. and Sato, R. (1967). *Methods Enzymol.* **10**, 536.

Ozols, J. (1986). *J. Biol. Chem.*, **261**, 3965.

Tamburini, P. P., Masson, H. A., Bains, S. K., Makowski, R. J., Morris, B. and Gibson, G. G. (1984). *Eur. J. Biochem.* **139**, 235.

Wittmann-Liebold, B., Salnikow, J. and Erdmann, V. A. (1986). in *Advanced Methods in Protein Microsequence Analysis*, p. 77, Springer-Verlag, Berlin.

Yasukochi, Y. and Masters, B. S. S. (1976). *J. Biol. Chem.* **251**, 5337.

Received 24 May 1990; accepted 8 June 1990.