Pergamon

Biochemical Pharmacology, Vol. 47, No. 8, pp. 1457–1460, 1994.
Copyright © 1994 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0006-2952/94 $6.00 + 0.00

0006-2952(93)E0109-K

Evidence for a new cytochrome P450 form induced by 3-methylcholanthrene in rats

(*Received* 26 *November* 1993; *accepted* 3 *December* 1993)

Abstract—Evidence is presented for a new 3-methylcholanthrene (3MC)-induced form of cytochrome P450, P450MCX, in rat liver microsomes. P450MCX was co-purified with CYP1A1 from 3MC-treated male Sprague–Dawley rats but was resolved by gel electrophoresis. The M_r of P450MCX (56,700) was intermediate between CYP1A1 (57,000) and CYP1A2 (54,800). Monoclonal antibodies showed that P450MCX was immunorelated to both CYP1A1 and CYP1A2 but not to CYP2B1, CYP2C6 or CYP3A1. Immunoreactive P450MCX was not detectable in liver microsomes from untreated rats but was highly induced by 3MC and Aroclor 1254, although not induced by isosafrole. The N-terminal amino acid sequence of P450MCX, obtained from an electroblotted sample resolved on SDS–PAGE, did not match any known cytochrome P450 or other protein. P450MCX may be a new member of the CYP1 family.

Key words: liver; microsomes; drug metabolism; purification; CYP1A

The rat cytochrome P450CYP1 gene family presently comprises only two members, CYP1A1 and CYP1A2, both of which are inducible by PAH* [1, 2]. At least 15 other non-P450 proteins are concomitantly induced with CYP1A1 and/or CYP1A2 by 3MC or other PAH, such as TCDD [3]. Here we report the co-purification with rat CYP1A1 of a novel form of 3MC-induced P450, plus a method for the N-terminal amino acid sequencing of individual P450 forms in semi-purified preparations.

Materials and Methods

Chemicals. Alkoxyresorufins were synthesized as described elsewhere [4]. Resorufin was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). CHAPS, cholate, lecithin, 3MC, NADPH and molecular mass markers (SDS-6H high molecular mass standard mixture) were bought from the Sigma Chemical Co. (Poole, U.K.). PB, sodium salt, was bought from BDH Ltd (Poole, U.K.). Olive oil was BP grade, from Boots Ltd (Nottingham, U.K.). Immobilon-P membranes were bought from Millipore U.K. Ltd (Watford, U.K.). Emulgen 911 was a generous gift from the Kao Corporation (Tokyo, Japan). ISF and Aroclor 1254 were provided by Dr C. R. Elcombe, Zeneca Central Toxicology Laboratories, Alderley Park, U.K.

Animals, induction and microsome preparation. Adult (230–260 g), male Sprague–Dawley rats, bred in the University of Aberdeen, were induced with 3MC (80 mg/kg, i.p., as a 1% solution in olive oil, once, 3 days before death) and hepatic microsomes were prepared as described previously [5].

P450 purification. The cytochrome P450 forms, CYP1A1 and CYP1A2, and NADPH-cytochrome P450 (cytochrome *c*) reductase were purified from liver microsomes of 3MC- and PB-induced rats, respectively, by column chromatography as described previously [6]. Column effluent fractions were continuously monitored at 280 nm for protein and 405 nm for haem and assessed for protein purity by SDS–PAGE. Contiguous fractions showing both a high purity of 48,000–60,000 M_r protein bands and significant 405 nm absorption were pooled and assayed for total P450 and EROD and MROD activities. All concentrations of chemicals in purification buffers are v/v unless indicated otherwise.

Enzyme assays and immunoblotting. NADPH-supported EROD and MROD activities of liver microsomes and P450-containing fractions obtained at various stages during purification were determined at 37° in fluorimeter cuvettes as described previously [6]. Total P450 concentrations were measured spectrophotometrically as described elsewhere [7]. Protein concentrations were measured by a dye-binding method [8] as described previously [6]. Protein samples were electrophoresed and immunoblotted as described elsewhere [9], using a 12.5% resolving gel and 6 hr gel running time and previously characterized monoclonal antibodies to individual, purified forms of rat and human P450 [9, 10]. For the determination of M_r values on immunoblots, molecular weight marker proteins were transiently stained on the nitrocellulose sheets using Ponceau red dye and their positions marked with a scalpel blade.

N-terminal amino acid "blot-sequencing". The purified preparations containing CYP1A1 and CYP1A2, respectively, were dialysed against 10 mM phosphate buffer, pH 7.6 containing 15% glycerol, then "blot-sequenced" using a method described previously [11]. Briefly, the individual proteins in the dialysed column fractions were resolved by SDS–PAGE, electroblotted onto Immobilon-P membranes, visualized by staining with Coomassie Blue, individually cut from the membrane with a scalpel and loaded into the sample cell of an Applied Biosystems 470A gas-phase protein sequencer fitted with a 120A on-line PTH (phenylthiohydantoin) analyser. N-terminal amino acid sequencing of the excised protein bands was performed using automatic Edman degradation with the standard 03R PTH programme.

Results and Discussion

Since rat CYP1A1 and CYP1A2 show substrate selectivity for EROD and MROD, respectively [12, 13], the purification of CYP1A1 was indicated by an increase in EROD accompanied by a decrease in MROD activity in column fractions. The identities of CYP1A1 and CYP1A2 in the final purified preparations were confirmed by N-

* Abbreviations: ARO, Aroclor 1254; CHAPS, 3-[(3-cholamidopropyl) - dimethylammonio] - 1 - propanesulphonate; DTT, dithiothreitol; EROD, ethoxyresorufin *O*-deethylase; ISF, isosafrole; MROD, methoxyresorufin *O*-demethylase; 3MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbons; PB, phenobarbitone; PCN, pregnenolone 16α-carbonitrile; P450, cytochrome P450.

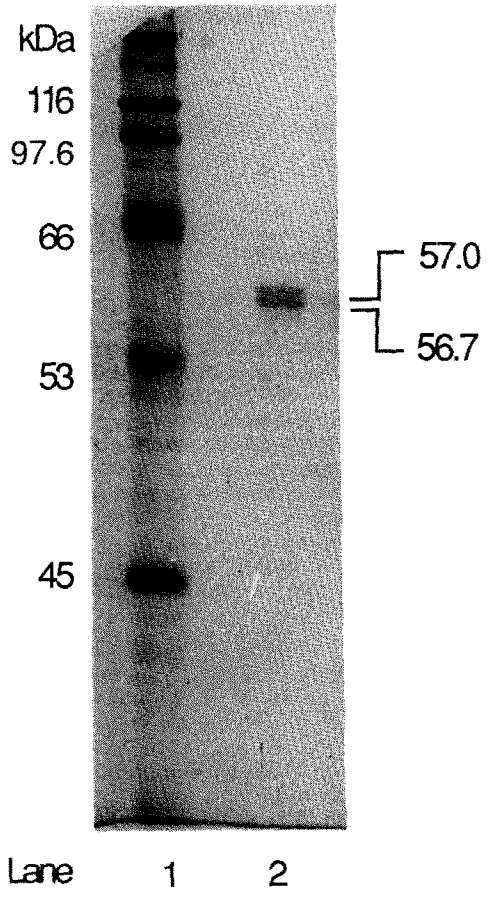

Fig. 1. SDS–PAGE of purified CYP1A1 preparation. The preparation was loaded at 2 μg protein per lane and run as described in Materials and Methods. Lane 1, molecular weight markers. Lane 2, purified CYP1A1 preparation.

terminal amino acid "blot-sequencing". CYP1A1 eluted from octylamino-Sepharose as a single peak in 10 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.35% cholate and 0.16% Emulgen 911. The pooled fractions comprising the first half of this peak had the highest EROD:MROD ratio (10:1) and a total P450 specific content = 7–9 nmol/mg protein and were further purified on DEAE Tris–acryl. CYP1A1 eluted as a single peak between 30–80 mM KCl in 10 mM HEPES buffer, pH 7.8, containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.05% CHAPS and 0.1% Emulgen 911. The pooled fractions comprising the first half of this peak had the highest EROD:MROD ratio (27:1) and a total P450 specific content = 10 nmol/mg protein and were further purified on S-Sepharose. CYP1A1 eluted as a single peak, henceforth called the purified CYP1A1 preparation (EROD:MROD ratio = 89:1, total P450 specific content = 16.6 nmol/mg protein), between 125–200 mM KCl in 10 mM potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.05% CHAPS and 0.1% Emulgen 911. CYP1A2 eluted from octylamino-Sepharose as the second half of the peak referred to above in 0.16% Emulgen and was further purified on Q-Sepharose to a total specific P450 content = 12 nmol/mg protein. The purified CYP1A1 preparation appeared as a single band (M_r = 57,000) on SDS–PAGE when electrophoresed as normal for 4 hr using a 10% resolving gel. However, this apparently homogeneous CYP1A1 band was split into two protein bands, with M_r = 57,000 and 56,700, respectively, by extending the period of electrophoresis to 6 hr and using a 12.5% resolving gel (Fig. 1). For comparison, purified CYP1A2 gave an M_r value = 54,800 and the 57,000 and 56,700 bands were absent even using the extended SDS–PAGE conditions (data not shown). The degree of purification of the CYP1A1 preparation over the original microsomes was 10-fold based on total P450 (total P450 specific content = 16.6 and 1.6 nmol/mg protein for the purified preparation and microsomes, respectively) and 12-fold based on the EROD activity (233 and 19 nmol/min/mg protein for the purified preparation and microsomes, respectively). The EROD:MROD ratio was 15-fold higher in the purified CYP1A1 preparation (89:1) than the microsomes (6:1), since whereas EROD activity increased 12-fold during the purification, MROD activity decreased slightly (3.3 and 2.6 nmol/min/mg protein for microsomes and purified CYP1A1 preparation, respectively). The EROD and MROD turnover numbers of the purified CYP1A1 preparation (EROD and MROD = 20 and 0.2 nmol/min/nmol P450, respectively) were similar to values previously reported for purified CYP1A1 [14]. An attempt to separate the two proteins by further chromatography on octyl-Sepharose with elution by an Emulgen 911 step gradient was unsuccessful.

Based on the relative visual intensities of Coomassie Blue staining on SDS–PAGE, the 57,000 and 56,700 proteins in the purified CYP1A1 preparation were considered to be present in approximately equal amounts. Since no other Coomassie-stained protein bands were visible and the total P450 specific content was close to the theoretical maximum (i.e. there was little or no non-P450 protein present), it was considered that both the proteins were probably cytochromes P450.

In order to identify the 57,000 and 56,700 proteins by N-terminal amino acid sequencing, advantage was taken of the ability to separate them on SDS–PAGE. The resolved proteins were electroblotted onto an Immobilon-P membrane and individually N-terminal sequenced. An SDS–PAGE loading of approximately 30–40 pmol total P450 of the purified CYP1A1 preparation provided

Table 1.

M_r	Form	Residue No. 5 10 15
57,000	CYP1A1	P S V Y G F P A F T S A T E L L L A
56,700	P450MCX	M F L P G I L L L L L L W
54,800	CYP1A2	A F S Q Y I S L A P E L X L A

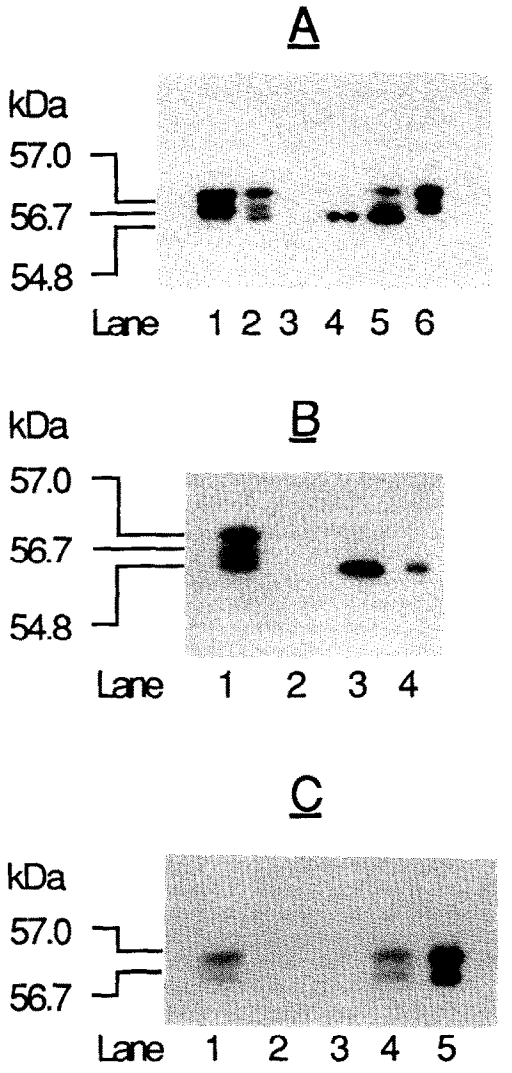

Fig. 2. Immunoblots of P450MCX, CYP1A1 and CYP1A2. Samples were resolved by SDS–PAGE and immunoblotted as described in Materials and Methods. Microsomes and purified P450s were loaded at 4.0 and 0.5–1.0 μg protein per lane, respectively. (A) Blotted with antibody RM1. Lanes 1 and 6, purified CYP1A1 preparation (1.0 and 0.8 μg protein loading, respectively). Lanes 2–5, liver microsomes from 3MC-treated, untreated, ISF- and Aroclor-treated rats, respectively. (B) Blotted with antibody RM1. Lanes 1–3, liver microsomes from 3MC-treated, untreated and ISF-treated rats, respectively. Lane 4, purified CYP1A2 (0.5 μg protein loading). (C) Blotted with antibody RM3. Lanes 1–4, liver microsomes from 3MC-treated, untreated, ISF- and Aroclor-treated rats respectively. Lane 5, purified CYP1A1 preparation (0.8 μg protein loading).

sufficient protein for the sequencing. A similar method was used to sequence purified CYP1A2. Single N-terminal amino acid sequences were obtained for the 57,000 and 56,700 proteins and purified CYP1A2 as shown in Table 1.

The sequences obtained for CYP1A1 and CYP1A2 exactly match those published [15], assuming that the terminal methionine predicted by their cDNA sequences had been cleaved. The sequence obtained for the 56,700, however, did not match any protein in the SWISSPROT database. Since there was no identity between the sequence for the 56,700 protein and the published sequences of CYP1A1 and CYP1A2, it is most unlikely that the 56,700 protein was a breakdown product of either of these P450s. Based on the unique sequence for this protein, plus the deduction (see above) that it was P450, we concluded that the 56,700 protein was a hitherto unreported form of P450, to which we have given the trivial name P450MCX (i.e. an unknown 3MC-induced P450). Interestingly, the nearest sequence match for P450MCX was to the extreme N-terminus of CYP21A1 (P450 steroid 21-hydroxylase, EC 1.14.99.10), having only three conservative alterations within the 13 amino acids sequenced (F/L, I/L and W/L at positions 2, 6 and 13, respectively).

Three monoclonal antibodies (RM1, RM2 and RM3) raised against the purified CYP1A1 preparation all recognised two protein bands, assumed to be CYP1A1 and P450MCX, on immunoblots of the purified CYP1A1 preparation (Fig. 2A, lanes 1 and 6; Fig. 2C, lane 5; data not shown for antibody RM2). Antibody RM1 also recognised purified rat CYP1A2 (Fig. 2B, lane 4), but antibodies RM2 and RM3 did not (data not shown). Antibody RM1 recognised 3 bands, considered to be CYP1A1, P450MCX and CYP1A2, respectively (in order from top to bottom), on immunoblots of 3MC- and Aroclor-induced rat liver microsomes (Fig. 2A, lanes 2 and 5, respectively). On immunoblots of ISF-induced rat liver microsomes, however, antibody RM1 recognised only a single band (Fig. 2A, lane 4; Fig. 2B, lane 3), which corresponded in M_r value to the lowest band in 3MC- and Aroclor-induced rats and to purified CYP1A2 (Fig. 2B, lane 4). Note that the highest band (CYP1A1) was the most intense in 3MC-induced rats whereas the lowest band (CYP1A2) was the most intense in Aroclor-induced rats. No bands were recognised by any of the three antibodies on immunoblots of liver microsomes from untreated rats at the protein loading used (Fig. 2A, lane 3 and Fig. 2C, lane 2; data not shown for antibody RM2). Antibody RM3, which did not recognise purified CYP1A2, did not recognise any band in ISF-induced microsomes (Fig. 2C, lane 3) and recognised only the two highest of the three bands (CYP1A1 and P450MCX) in 3MC- and Aroclor-induced microsomes (Fig. 2C, lanes 1 and 4). Antibody RM1 did not recognise any proteins on immunoblots of liver microsomes from PB- or PCN-induced rats (data not shown). Monoclonal antibodies which specifically recognised CYP2B1, CYP2C6 and CYP3A1 (plus CYP3A2), respectively, did not recognise any proteins on immunoblots of the purified CYP1A1 preparation, purified CYP1A2 or liver microsomes from 3MC-induced rats (data not shown).

In conclusion, the evidence presented here indicates the presence of a previously unreported 3MC-inducible form of P450, P450MCX, which is not apparently constitutive (or is expressed normally at very low levels) and is also induced by Aroclor but not by ISF, PB or PCN. Although its N-terminal amino acid sequence was distinct from CYP1A1 and CYP1A2, the fact that three monoclonal antibodies apparently specific to the CYP1A subfamily [10] all recognised both P450MCX and CYP1A1 indicates that P450MCX is probably a member of the CYP1 family. Although only two members of the CYP1 family are currently recognised in any species, allelic variation in human CYP1A1 has been reported [16], but P450MCX is almost certainly not an allelic variant of CYP1A1 or CYP1A2. Despite over two decades of intensive research on P450, novel forms probably yet await discovery. For example, a new PAH-inducible P450 (P450-EF), which is distinct from CYP1A1 and CYP1A2 and has an M_r (55,000) similar to P450MCX and likewise lower than CYP1A1 has been described, but without a sequence [17]. Another novel P450 which is recognised by monoclonal antibodies

to CYP1A1/CYP1A2 is induced in rat liver by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), but has not been sequenced [18]. However, the M_r of the PhIP-induced protein (51,000) is less than those of both CYP1A1 and CYP1A2 in the same paper (56,000 and 54,000, respectively) [18], whereas the M_r of P450MCX was intermediate between CYP1A1 and CYP1A2. Three lines of evidence suggest that P450MCX is more closely related, both in structure and regulation, to CYP1A1 than to CYP1A2: (i) P450MCX and CYP1A1 co-purified under conditions which separated CYP1A2; (ii) two of the monoclonal antibodies which recognised both P450MCX and CYP1A1 did not also recognise CYP1A2; and (iii) with the regimen used, both P450MCX and CYP1A1 were induced by 3MC but not ISF, whereas CYP1A2 was induced by both chemicals.

Acknowledgement—R.J.W. was an SERC-CASE PhD student with the Wellcome Foundation Ltd.

*Departments of *Biomedical Sciences and †Molecular and Cell Biology University of Aberdeen Marischal College Aberdeen AB9 1AS, U.K.*
‡Department of Bioanalysis and Drug Metabolism The Wellcome Foundation Ltd Beckenham Kent BR3 3BS, U.K.

RICHARD J. WEAVER*
BRYAN DUNBAR†
MAURICE DICKINS‡
WILLIAM T. MELVIN†
JOHN FOTHERGILL†
M. DANNY BURKE*§

REFERENCES

1. Gonzalez FJ, Molecular genetics of the P-450 superfamily. *Pharmacol Ther.* **45**: 1–38, 1990.
2. Funae Y and Imaoka S, Cytochrome P450 in rodents. In: *Handbook of Experimental Pharmacology* (Eds. Schenkman JB and Greim H), pp. 221–238. Springer-Verlag, Berlin, 1993.
3. Nebert DW and Gonzalez FJ, P450 genes: structure, evolution and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.
4. Burke MD and Mayer RT, Differential effects of phenobarbitone and 3-methylcholanthrene induction on the hepatic microsomal metabolism and cytochrome P450-binding of phenoxazone and a homologous series of its *n*-alkyl ethers (alkoxyresorufins). *Chem Biol Interact* **45**: 243–258, 1983.
5. Burke MD, Falzon M and Milton AS, Decreased hepatic microsomal cytochrome P450 due to indomethacin: protective roles of 16,16-dimethylprostaglandin F2α and inducing agents. *Biochem Pharmacol* **32**: 389–397, 1983.
6. Weaver RJ, Dickins M and Burke MD, Cytochrome P4502C9 is responsible for hydroxylation of the naphthoquinone antimalarial drug 58C80 in human liver. *Biochem Pharmacol* **46**: 1183–1197, 1993.
7. Omura T and Sato R. The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
8. Schaffner W and Weissmann C, A rapid and sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem* **56**: 502–514, 1973.
9. Shaw PM, Barnes TS, Cameron D, Engeset J, Melvin WT, Omar G, Petrie JC, Rush WR, Snyder CP, Whiting PH, Wolf CR and Burke MD, Purification and characterization of an anticonvulsant-induced human cytochrome P-450 catalysing cyclosporin metabolism. *Biochem J* **263**: 653–663, 1989.
10. Murray GI, Foster CO, Barnes TS, Weaver RJ, Snyder CP, Ewen SWB, Melvin WT and Burke MD, Cytochrome P450IA expression in adult and fetal human liver. *Carcinogenesis* **13**: 165–169, 1992.
11. Horak A, Dunbar B, Fothergill JE and Wilson SB, The plant mitochondrial F1-ATPase. *FEBS Lett* **274**: 9–11, 1990.
12. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
13. Namkung MJ, Yang HL, Hulla JE and Juchau MR, On the substrate specificity of cytochrome P450IIIA1. *Mol Pharmacol* **34**: 628–637, 1988.
14. Wolf CR, Seilman S, Oesch F, Mayer RT and Burke MD, Multiple forms of cytochrome P-450 related to forms induced marginally by phenobarbital. *Biochem J* **240**: 27–33, 1986.
15. Soucek P and Gut I, Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica* **22**: 83–103, 1900.
16. Hayashi S-I, Watanabe J, Nakachi K and Kawajiri K, Genetic linkage of lung cancer-associated Msp1 polymorphisms with amino acid placement in the heme binding region of the human cytochrome P4501A1 gene. *J Biochem* **110**: 407–411, 1991.
17. Christou M, Keith IM, Schroeder ME and Jefcoate C, Reversal of cytochrome P-4501A1 and P-450-EF expression in MCA-C3H/10T1/2 cell-derived tumours as compared to cultured cells. *Cancer Res* **53**: 968–976, 1993.
18. Adachi H, Degawa M, Miura S-I, Hashimoto Y, Sugimura T and Esumi H, Induction of a putative new cytochrome P450 isozyme in rat liver by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Biochem Biophys Res Commun* **174**: 797–803, 1991.

§ Corresponding author.