ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/21429867

Aminopyrine-N-demethylase. II. Characterization of a unique monooxygenase isoform P-450ap

ARTICLE *in* EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS · JULY 1991

Impact Factor: 1.56 · DOI: 10.1007/BF03189962 · Source: PubMed

CITATION
1

READS
5

2 AUTHORS, INCLUDING:

Ilya Tsyrlov
XENOTOX, Inc.
219 PUBLICATIONS **424** CITATIONS

SEE PROFILE

Available from: Ilya Tsyrlov
Retrieved on: 25 January 2016

EUROPEAN JOURNAL OF DRUG METABOLISM AND PHARMACOKINETICS, 1991, Vol. 16, No. 3, pp. 213–217

Aminopyrine-N-demethylase. II. Characterization of a unique monooxygenase isoform P-450ap

I.B. TSYRLOV and K.E. GERASIMOV

Laboratory of Xenobiochemistry, Institute of Clinical and Experimental Medicine, Academy of Medical Sciences, Siberian Division, Novosibirsk, USSR

Received for publication : February 16, 1990

Keywords : 4-isopropylaminoantipyrine, cytochrome P-450, N-demethylation

SUMMARY

A previously unidentified cytochrome P-450ap possessing the highest aminopyrine-N-demethylase activity has been isolated from liver microsomes of 4-isopropylaminoantipyrine-induced rats, using affinity chromatography in combination with ion-exchange chromatography with subsequent separation on a hydroxyapatite column. The isolated cytochrome P-450ap has the following characteristics: Mr = 49 kD, CO-peak maximum at 450.5 nm, rate of demethylation in a reconstituted system for aminopyrine of 25.5 nmoles of HCHO/min per nmole of P-450, and for benzphetamine a rate of 17.0nmoles of HCHO/min per nmole of P-450. The hemoprotein synthesis is paralleled by the synthesis of a protein with Mr of 51 kD. Immunochemical analysis permitted the identification of the latter protein as cytochrome P-450$_b$. It was, demonstrated that cytochrome P-450ap does not interact with the antibodies to the major phenobarbital induced form, i.e. with cytochrome P-450$_b$.

INTRODUCTION

The microsomal monooxygenase system involved in metabolism of endogenous (steroid hormones, prostaglandins) and exogenous (xenobiotics) substrates can be induced by compounds of different chemical structures (1). The major component of this system – cytochrome P-450 – exists in multiple, at least twenty, molecular forms differing in substrate specificity and a whole set of spectral, electrophoretic, catalytic, and immunochemical properties (2).

Phenobarbital (PB) represents one of the major classes of inducers. It induces the synthesis of two structurally and immunologically related forms of cytochrome P-450: cytochrome P-450 with extremely low enzymic activity (3), and P-450$_b$, which plays a key role in the detoxification of most ingested xenobiotics, including drugs (4).

It was previously thought that it is the major PB-inducible form, cytochrome P-450$_b$, that possesses aminopyrine (AP) N-demethylase activity, as well as benzphetamine (BP) N-demethylase activity (5). However, different patterns of inhibition by antibodies against cytochrome P-450$_b$, the demethylation of AP and BP in 4-isopropylaminoantipyrine (IPAAP) microsomes and also the presence of a second *de novo* synthesized polypeptide of 49 kD (6) in these microsomes prompted our attempts to isolate individual forms of cytochrome P-450, the catalyzer of AP metabolism.

Cytochrome P-450 isoforms were isolated from the liver microsomes of rats given IPAAP. This was done to compare the catalytic, spectral, electrophoretic and immunological properties of the isolated demethylase of AP with those of cytochrome P-450$_b$ from the PB-induced microsomes.

Please send reprint requests to : Dr I.B. Tsyrlov, Laboratory of Xenobiochemistry, Institute of Clinical and Experimental Medicine, Academy of Medical Sciences, Siberian Division, 630117 Novosibirsk, USSR

MATERIALS AND METHODS

IPAAP was synthesised in the Institute of Organic Chemistry (Novosibirsk, USSR). PB was obtained from Sigma (USA); 1,8-diaminooctane from Merck (Germany); BrCN-activated Sepharose 4B, DEAE-Sephacel from Pharmacia (Sweden); hydroxyapatite from BioRad Labs (USA); NADPH from Boehringer Mannheim (Germany). All the rest of the reagents used for this study were of analytical grade.

Male, Wistar rats (180–200 g) fed on standard laboratory diet and given water *ad libitum* were used throughout. Animals were pretreated with IPAAP and PB received 80 mg/kg i.p. for four consecutive days.

Isolation of liver microsomal fraction, determination of the contents of microsomal protein and cytochrome P-450, Ouchterlony double diffusion reactions, rocket immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis were carried out as described in the preceding paper (6). Spectral studies were performed using a double wavelength double beam spectrophotometer Hitachi-557 (Japan).

Fractions of cytochrome P-450 induced by IPAAP or PB were isolated as described by Guengerich and Martin (7) with subsequent separation on a hydroxyapatite column.

Enzyme activities were determined using a reconstituted system, containing 0.1 nmole of cytochrome P-450, 650 units (1 unit = the amount of NADPH–cytochrome P-450 reductase necessary for the reduction of 1 nmole of cytochrome c per min) of NADPH–cytochrome P-450 reductase and 15 g of dilauroylphosphatidylcholine in 1 ml of phosphate buffer.

Immunoinhibitory analysis was carried out according to Kamataki (8).

RESULTS

By combining affinity and ion-exchange chromatography we separated some cytochrome P-450-containing fractions from the IPAAP-microsomes. The number of separated fractions was three (Fig. 1). Fractions I and II were composed of several proteins with a molecular weight of 48–52 kD, whereas fraction III was electrophoretically homogen- ous with a molecular weight of 51 kD (Fig. 2). Fraction III was similar in catalytic and spectral properties to cytochrome P-450$_b$ (Table I). In the Ouchterlony double immunodiffusion reaction, when antibodies against PB-induced cytochrome P-450$_b$ (anti-P-450$_b$) were used, fraction III showed a distinct

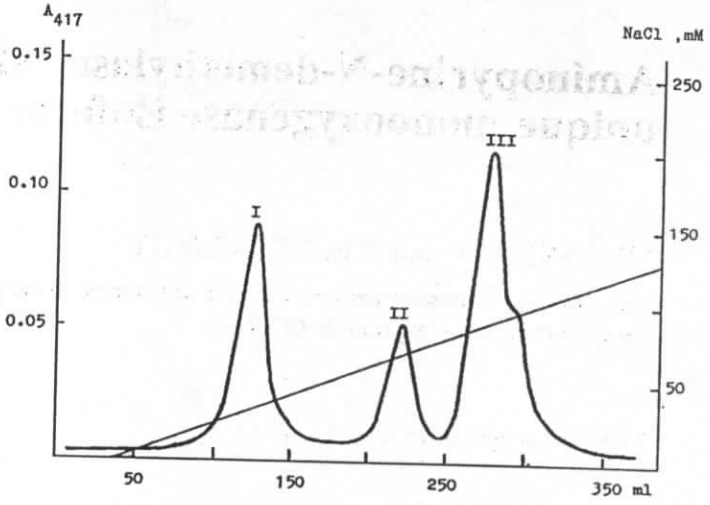

Fig. 1 DEAE-Sephacel column chromatography

immunoprecipitin band. It should be noted that fraction III was the only one cross-reacting with these antibodies, the other fractions isolated after ion-exchange chromatography gave no precipitin bands (Fig. 3). These data are supported by the results of inhibitory analysis of the AP- and BP-N-demethylation reactions catalyzed by cytochromes P-450 from fractions I, III and cytochrome P-450$_b$. It was shown

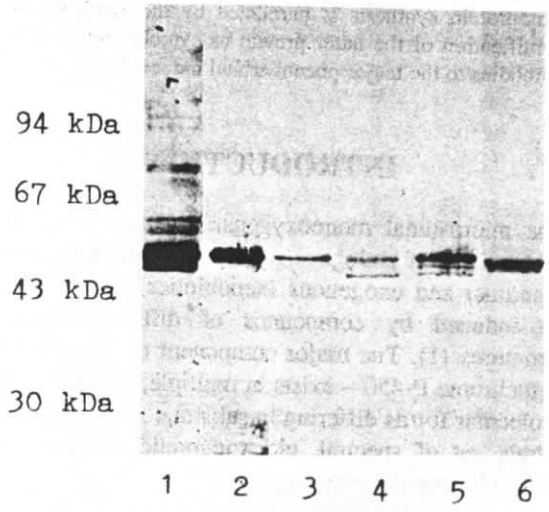

Fig. 2 SDS polyacrylamide gel electrophoresis of microsomes preinduced by IPAAP and cytochrome P-450 fractions
1 – microsomes (20 μg)
2 – fraction I (2 μg)
3 – fraction III (1 μg)
4 – fraction HAP-1 (1 μg)
5 – fraction HAP-2 (2 μg)
6 – fraction HAP-3 (2 μg)

Table I Characteristics of microsomes and cytochromes P-450, induced by IPAAP and PB

Fractions	*CO-peak maximum (nm)*	*N-demethylation rate**	
		AP	*BP*
IPAAP-microsomes	450.0	10.5	18.0
Fraction I	450.0	11.8	15.8
Fraction II	449.5	1.2	5.0
Fraction III	450.0	12.0	41.6
HAP-1	450.0	12.5	15.0
HAP-2	449.8	14.4	12.5
HAP-3	450.5	25.5	17.0
Cytochrome P-450$_b$	450.0	17.2	62.5
Untreated microsomes	450.0	3.4	5.2

*nmoles of HCHO/min per mg of protein (for microsomes), nmoles of HCHO/min per nmole of P-450 (for isolated cytochromes P-450)

that anti-P-450$_b$ is equally efficient in decelerating these reactions in a reconstitution system containing cytochrome P-450$_b$ or P-450 of fraction III, and that it has no effect on the metabolism of these substrates by forms of cytochrome P-450 in fraction I (Fig. 4). The results obtained allowed us to identify one of the isolated cytochrome P-450 forms, i.e. to identify fraction III as cytochrome P-450$_b$.

Fraction I showed the higher AP-N-demethylation activity, 11.8 nmoles of HCHO/min per nmole of P-450, while the N-demethylation rate of BP remained low compared to that of cytochrome P-450$_b$. It is noteworthy that the cytochrome P-450 fraction with the highest AP-N-demethylation activity appeared either at the start of the salt gradient (ion-exchange chromatography) or in the void volume, whereas the major fraction III was eluted in the middle of the gradient (0.08–0.12 M) that is a feature of the chromatographic behaviour of PB-cytochrome P-450$_b$ (7).

The presence of proteins with different molecular weights in fraction I encouraged us to undertake its further purification through a hydroxyapatite column. As a result, we isolated three cytochrome P-450 fractions: HAP-1, HAP-2, HAP-3 (Fig. 5). All these fractions oxidized BP at the same rate, but differed in the oxidation rate of AP (Table I). The N-demethylation rate of this substrate in fraction HAP-3 was much higher than in the other fractions. This finding allowed us to identify the isolated hemoprotein as an individual form of cytochrome P-450ap highly specific for AP.

Cytochrome P-450ap was electrophoretically represented as a protein with a molecular weight of 49 kD. Radioisotopic analysis of the IPAAP-microsome preparations was carried out after their electrophoretic separation. The results of this analysis indicated that the induction of microsomal monooxygenases by IPAAP is associated with a *de novo* synthesis of two different cytochromes P-450. Thus two bands appear on the fluorogram, one with a

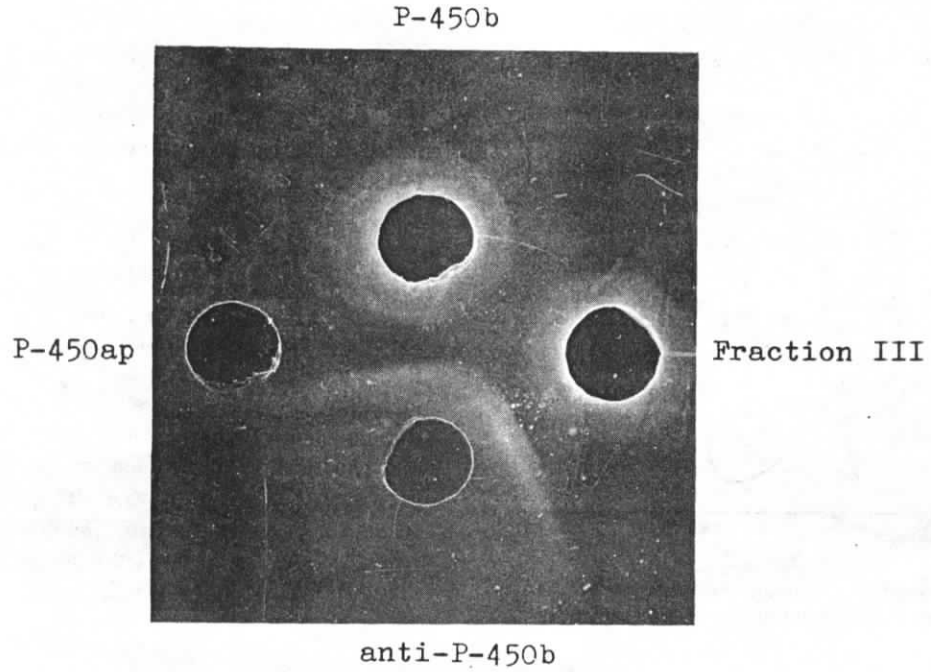

Fig. 3 Ouchterlony double diffusion analysis

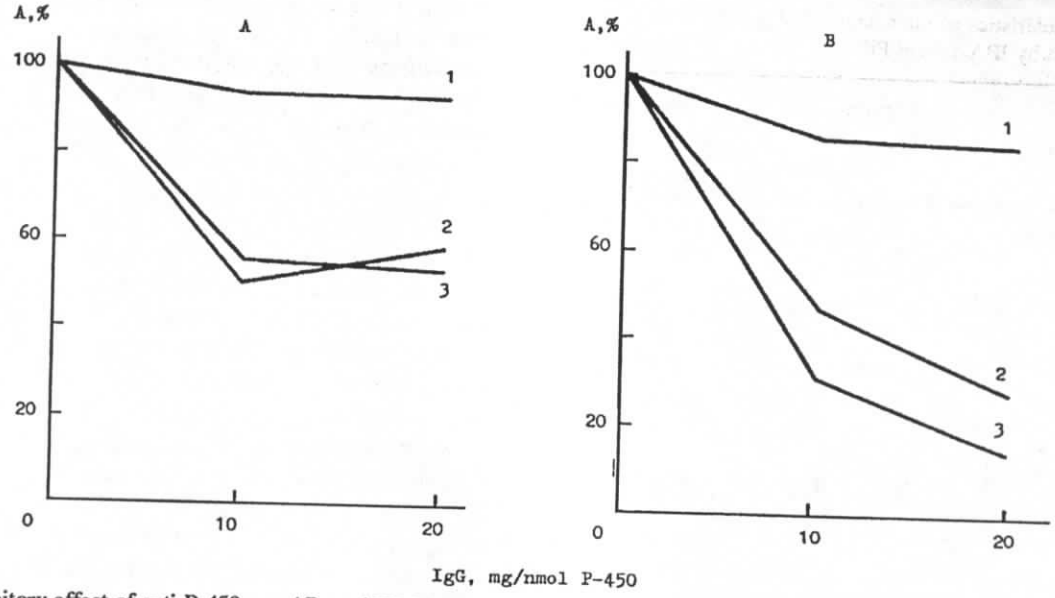

Fig. 4 Inhibitory effect of anti-P-450$_b$ on AP- and BP-N-demethylation in reconstitutive system
1 – fraction I
2 – cytochrome P-450$_b$
3 – fraction III
100% – rate of AP- and BP-N-demethylation reactions in presence of control rabbit immunoglobulins

molecular weight of 51 kD representing cytochrome P-450$_b$, another with a molecular weight of 49 kD representing AP-N-demethylase – P-450ap (6).

From the analysis of spectral characteristics it was concluded that the heme iron of the isolated cytochromes P-450 was in a low spin state. The maximum of the CO-peak of cytochrome P-450ap was shifted by 0.5 nm in the long wave range. This makes it different from the other cytochromes P-450,

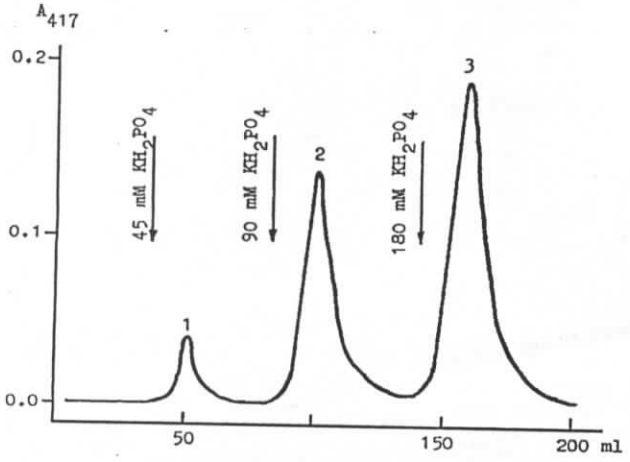

Fig. 5 Hydroxyapatite column chromatography of cytochromes P-450 from fraction I (step gradient of KH_2PO_4)
1 – fraction HAP-1
2 – fraction HAP-2
3 – fraction HAP-3

including P-450$_b$. This difference was manifest in the Ouchterlony test. Indeed, no precipitin bands appeared when cytochrome P-450ap reacted with anti-P-450$_b$ (Fig. 3).

The results of rocket immunoelectrophoresis with the use of antibodies against cytochrome P-450ap provided evidence indicating that the relative content of the cytochrome P-450ap in IPAAP-induced rat liver microsomes amounts to 20% of the total CO-binding hemoprotein. The total concentration of the monooxygenase isozymes, namely P-450$_b$ and P-450ap in these microsomes, has been shown to reach 56% of the total P-450 content.

DISCUSSION

Investigation of the multiple forms of cytochrome P-450 induced in the organism by various xenobiotics, including drugs, is very helpful in identification of the substrate specificity of microsomal monooxygenases and clarification of mechanisms of detoxifying liver processes (2). The choice of appropriate specific substrates is, so far, limited. The previous experiments with the liver microsomal fraction have demonstrated that AP-N-demethylation is induced in response to PB administration (5) and, as a rule, the response is correlated with an increase in BP-N-demethylase activity. In fact, we obtained fraction III when IPAAP was used as an inducer. Fraction III metabolizes AP

with a rate close to the one of cytochrome P-450$_b$. Fraction III also possesses high BP-N-demethylation activity comparable to that of PB-preinduced cytochrome P-450$_b$. Judging by its spectral, catalytic, electrophoretic and immunochemical properties, fraction III may with good reason be regarded as an individual form of monooxygenase – cytochrome P-450$_b$.

The fraction of cytochrome P-450 named HAP-3 deserves special comment. Of all the fractions, it is distinguished by the highest rate of N-demethylation of AP, 25.5 nmoles of HCHO/min per nmole of P-450; in contrast, the N-demethylation rate of BP differed slightly from that of the other fractions studied and was markedly lower than that of cytochrome P-450$_b$.

The purpose of the present study was not only to determine the absolute values for the demethylase activities of the fractions obtained, but even more to determine the ratio of the demethylation rate of AP to that of BP (the latter is a substrate highly specific to cytochrome P-450$_b$). Thus, the ratio for PB-induced cytochrome P-450$_b$ was found to be 0.27, that for P-450$_b$ in fraction III – 0.28 but for cytochrome P-450 in fraction HAP-3 it was 1.3. We have called this monooxygenase form cytochrome P-450ap, because cytochrome in the fraction HAP-3 was characterized as having the highest demethylation activity for AP and not for BP which differed in this parameter from cytochrome P-450$_b$ induced by either PB or IPAAP.

Thus, we have isolated an individual form of cytochrome P-450 from rat liver microsomes and given its characterization. This form has a high AP-N-demethylation activity that is not correlated with BP-N-demethylation activity. Support for this finding came from immunochemical analysis: antibodies against cytochrome P-450$_b$ did not cross react with cytochrome P-450ap.

It is of interest that other animal species, as well as rats, have a form of cytochrome P-450 with higher N-demethylase activity for AP compared to BP. For example, a constitutive monooxygenase form – cytochrome P-450 LM3b – has been isolated from the livers of rabbits. This monooxygenase form has the following catalytic characteristics: an AP-N-demethylation rate of 9.7 nmoles of HCHO/min per nmole of P-450, and a BP-N-demethylation rate of 5.9 nmoles of HCHO/min per nmole of P-450 (9).

We isolated and characterized the AP-N-demethylases from livers of mice treated with PB and 1,4-*bis*[2-(3,5-dichloropyridyl-oxy)]benzene exceeded the rate of AP metabolism by cytochrome P-450$_b$ by 2.3 and 1.3 times respectively (10).

All of these P-450 cytochromes, including P-450ap, in contrast to the major PB-form of monooxygenase, are usually eluted in the void volume during ion-exchange chromatography. They also have other properties in common in different species of animals.

Taken together, the results show that the administration of PB-type inducers to animals is consistently associated with the synthesis of cytochromes P-450$_b$ and P-450$_e$ in liver microsomes. This conclusion does not, however, exclude in a number of particular cases a *de novo* synthesis of a cytochrome P-450 specific to the given xenobiotic and different from cytochrome P-450$_b$. A case in point here is cytochrome P-450ap which we have isolated and characterized.

REFERENCES

1. Conney A.H. (1967) : Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev., 19, 317-366.
2. Nebert D.W., Negishi M. (1982) : Multiple forms of cytochrome P-450 and the importance of molecular biology and evolution. Biochem. Pharmacol., 31, 2311-2317.
3. Guengerich F.P., Dannan G.A., Wright S.T., Martin M.V., Kaminsky L.S. (1982) : Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. Biochemistry, 21, 6019-6030.
4. Ryan D.E., Thomas P.E., Reik L.M., Levin W. (1982) : Purification, characterization and regulation five rat hepatic microsomal cytochrome P-450 isozymes. Xenobiotica, 12, 727- 744.
5. Guengerich F.P., Dannan G.A., Wright S.T., Martin M.V., Kaminsky L.S. (1982) : Purification and characterization of microsomal cytochrome P-450s. Xenobiotica, 12, 701-716.
6. Tsyrlov I.B., Gerasimov K.E. (1991) : Aminopyrine-N-demethylase. I. Directed modification of substrate structure as a way of production of inducer of the monooxygenase isoform P-450$_b$. Eur. J. Drug Metab. Pharmacokinet., 16, 207-212.
7. Guengerich F.P., Martin M.V. (1980) : Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase and epoxide hydratase from a single preparation of rat liver microsomes. Arch. Biochem. Biophys., 205, 365-379.
8. Kamataki T., Belcher A.H., Neal R.A. (1976) : Studies of the metabolism of diethyl-*p*-nitrophenyl phosphorothionate (parathion) and benzphetamine using an apparently homogeneous preparation of rat liver cytochrome P-450. Mol. Pharmacol., 12, 921-932.
9. Bonfild C., Dalet-Beluehe I., Maurel P. (1982) : Induction by triacetyloleandomycin and partial purification of LM3 form cytochrome P-450 from rabbit liver microsomes. Biochem. Biophys. Res. Commun., 104, 1011-1017.
10. Duzhak T.G., Gutkina N.I., Tsyrlov I.B., Lyakhovich V.V. (1988) : A comparison of cytochrome P-450 forms induced by phenobarbital and 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene in mouse and rat liver. Biochemistry (USSR), 53, 188-195.