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IMMUNOCHEMICAL CHARACTERIZATION OF SOME MONOOXYGENASE ACTIVITIES IN LIVER MICROSOMES FROM UNTREATED AND PHENOBARBITAL-TREATED RATS

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SUMMARY

Two major forms of liver microsomal cytochrome P 450, one from untreated rats (P 450 A_2NI) and the other from phenobarbital-treated rats (P 450 B_2PB), were partially purified. Reconstitution of monooxygenase activities of purified enzymes and inhibition patterns of these activities by antibodies in microsomes gave the following results : 1) aniline hydroxylase activity is mainly supported by cytochrome P 450 A_2NI. This form is the major one in microsomes from control rats, but is also found at minute amounts in microsomes from phenobarbital-treated rats. It behaves as a constitutive form. 2) 4-nitroanisole- and benzphetamine-demethylase activities are mainly supported by cytochrome P 450 B_2PB which is predominant in phenobarbital-treated rats but is also present in control microsomes at low levels. 3) 4-nitroanisole-O-demethylase activity is less specific than benzphetamine-N-demethylase activity towards cytochrome P 450 B_2PB.

INTRODUCTION

Cytochrome P 450 plays important roles in the metabolism of a wide variety of endogenous and exogenous substances such as steroids, fatty acids and xenobiotics. Many authors have extensively studied the different forms of cytochrome P 450 in rats, and mainly the phenobarbital- and 3-methylcholanthrene-induced forms (1, 2, 3). Only few studies were devoted to the constitutive liver microsomal cytochromes P 450, with respect to their substrate specificities. Different forms of cytochrome P 450 have been isolated from hepatic microsomes of control rats and rabbits ; these forms were shown to be also present in phenobarbital-treated animals, but in lower amounts (4, 5, 6, 7, 8). In order to characterize cytochromes P 450 in untreated and phenobarbital-treated rats, we have 1) purified the major forms of cytochrome P 450 in each type of microsomes, 2) prepared antibodies against these forms, 3) tested the inhibitory effect of these antibodies on three monooxygenase activities, namely aniline hydroxylase, benzphetamine N demethylase and 4-nitroanisole O demethylase.

Previous results have shown that aniline hydroxylase is a "non-inducible" activity (9, 10, 11) whereas 4-nitroanisole-O- and benzphetamine N-demethylases are "phenobarbital-inducible" monooxygenase activities (10,

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11). The latter activity has been extensively studied and shown, by reconstitution experiments, to be supported by a purified phenobarbital-inducible form of cytochrome P 450 (2, 3, 12, 13, 14). The purpose of the present study was 1) to demonstrate that the major form of cytochrome P 450 in control microsomes is more specific of aniline hydroxylase activity ; 2) to demonstrate that the major form of cytochrome P 450 from phenobarbital-treated rats is more specific of 4-nitroanisole-O-demethylase activity ; 3) to confirm previous results, showing the specificity of the latter form towards benzphetamine N-demethylase activity.

MATERIAL AND METHODS

Purification procedure of cytochrome P 450_S : The purification procedure has been described previously (15) and is summarized in scheme 1. Hepatic microsomes from control and phenobarbital-treated rats were prepared, solubilized in 0.2 percent (w/v) sodium cholate and applied to an Octyl-Sepharose CL4B (Pharmacia Chemicals) column. After elution, cytochrome P 450-containing fractions were pooled and applied to a DEAE Cellulose (DE 52 Whatman) column, as described by Warner et al. (4). Two cytochrome P 450 heterogenous fractions called A and B were obtained. P 450 A and B fractions were finally flowed through a CM Cellulose (CM 52 Whatman) (16). In control microsomes, cytochrome P 450 A was separated into cytochromes P 450 A_1 and A_2 ; cytochrome P 450 A_2NI was the predominant form ; the cytochrome P 450 B fractions, found in low amounts, were heterogenous and not further purified. In microsomes from phenobarbital-treated rats, the minor fractions (cytochrome P 450 A) were separated into cytochromes P 450 A_1 and A_2 ; the main peak of major fractions (cytochrome P 450 B) was further purified and separated into B_1 and B_2 fractions ; cytochrome P 450 B_2PB was the predominant form. Final preparations of cytochromes P 450 were concentrated, dialyzed against a 100 mM sodium phosphate buffer pH 7.4, containing 20 percent (v/v) glycerol, treated twice by biobeads SM 2 and stored at - 80°C. NADPH cytochrome c reductase fractions eluted from the Octyl Sepharose column were pooled and purified on 2'5' ADP Sepharose (17).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) were performed as described by Laemmli (18).

Preparation of the antibodies : Female New Zealand white rabbits were immunized intradermally along the flanks at 20 sites either with 260 µg per rabbit of purified cytochrome P 450 B_2PB (specific activity : 22.4 nmol x mg^{-1} proteins) or 500 µg per rabbit of purified cytochrome P 450 A_2NI (specific activity : 11.8 nmol x mg^{-1} proteins). The immunogen was diluted in 0.9 % NaCl to 1 ml, and 1 ml of Freund's complete adjuvant (Grand Island Biological Company) was added. Every 10 days, the rabbits were boosted with the same quantity of antigen in Freund's complete adjuvant. 4 injections were performed, and one week after the last boost, the rabbits were bled and sera were collected. The immunoglobulin fractions were prepared from serum by a 50 % ammonium sulfate precipitation, and further purified by DEAE-Cellulose chromatography (DE 52 Whatman), using a 100 mM sodium phosphate buffer pH 6.8. Control IgG was isolated from control rabbit sera in an identical manner. The purity of the IgG fractions, verified by electrophoresis on cellulose acetate (100 mM tris-barbital buffer pH 8.8) was at least of 85 %.

Ouchterlony double-diffusion analysis : The immunodiffusion plates were made with 0.9 % agarose, 80 mM NaCl, 15 mM sodium azide dissolved in 1 M glycine buffer pH 7.4. The plates loaded with the antigens and the antibodies were incubated at room temperature in a humid atmosphere for at least 24 hours.

Enzymatic assays and inhibition of enzyme activities by antibodies
Drug metabolizing activities : aniline hydroxylation, 4-nitroanisole-O-demethylation and benzphetamine N-demethylation were assayed in microsomes as previously described (19) and in reconstituted systems using purified P 450 forms. For the latter, monooxygenase activities were measured after a 10 min incubation in the following medium : 0.16 mM dilauroyl phosphatidylcholine, 0.46 mM sodium cholate, 500 U x $nmol^{-1}$ cytochrome P 450 of NADPH cytochrome c reductase, 0.3 to 0.6 pM purified cytochrome P 450, 1 mM substrate and buffers. For the substrates aniline and 4-nitroanisole, the buffer used contained 100 mM sodium phosphate pH 7.4, 10 mM magnesium chloride and 20 percent (v/v) glycerol. For the substrate benzphetamine, the buffer used was the same without glycerol. Formaldehyde produced was measured according to Nash's method (20).

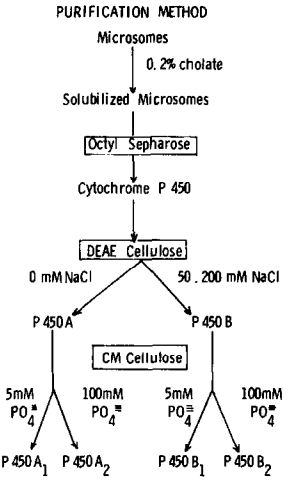
Inhibition of these three enzyme activities by antibodies was performed on microsomes. Microsomes were preincubated with various amounts of IgG from sera of immunized or non-immunized rabbits at 23°C for 10 min before the start of the reactions. The inhibitory effect of the antibodies was calculated using the catalytic activities in the presence of the same amount of control IgG as 100 percent.

Assays : Cytochrome P 450 (21), proteins (22) and NADPH cytochrome c reductase (19) were measured according to described methods.

RESULTS AND DISCUSSION

The main form of cytochrome P 450 in control microsomes is the P 450 A_2 form, called P 450 A_2NI (scheme 1). This form was obtained with a specific activity of 11.8 nmol x mg^{-1} proteins ; by SDS-PAGE, we observed one major band and two faint bands (data not shown), showing a slight contamination of the purified P 450 A_2NI. The anti P 450 A_2NI antibody reacts with the homologous antigen in a single precipitin band but reacts slightly with P 450B NI forms. The latter reaction might be due to either a cross-reaction, or a faint contamination of the anti P 450A_2NI antibody by an antibody against the P 450 BNI fraction. The second hypothesis seems more likely since the cytochrome P 450 used to immunize the rabbits was probably slightly contaminated by P 450 BNI forms. The anti P 450 A_2NI antibody does not recognize the cytochrome P 450 B_2PB (fig. 1A). The main form of cytochrome P 450 in microsomes from phenobarbital-treated rats is the B_2 form, called P450 B_2PB (scheme 1). This form was purified to homogeneity as shown by its specific activity (22.4 nmol x mg^{-1} proteins) and by SDS-PAGE (one single band)(data not shown). The anti P 450 B_2PB antibody reacts only with the cytochrome P 450 B_2PB in a single precipitin band, showing the high specificity of this antibody (Fig. 1B).

In reconstitution experiments, the cytochrome P 450 A_2NI supported a high aniline hydroxylase activity (1.1 nmol of p-aminophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450) and low benzphetamine and 4-nitroanisole demethylase activities (0.17 nmol of formaldehyde, 0.18 nmol of p-nitrophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450 respectively). Moreover, the cytochrome P 450 B_2PB in the same conditions, supported high benzphetamine- and 4-nitro-

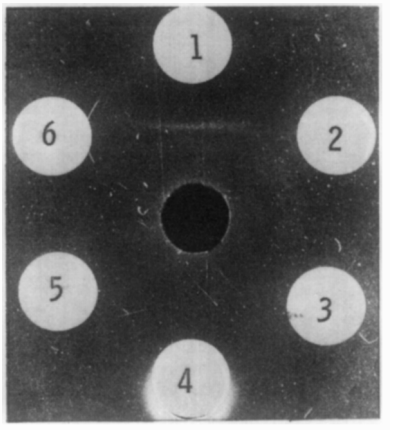


Scheme 1 : Purification method of cytochrome P 450_s

In control microsomes, at least two fractions of cytochrome P 450 B were eluted on DEAE cellulose. They were obtained in low amounts, pooled, called P 450 BNI and not further purified. In microsomes from phenobarbital-treated rats, one cytochrome P 450 B form, very predominant, was further purified and separated into B_1 and B_2.

anisole demethylase activities (7. 4 nmol of formaldehyde, 2.6 nmol of p-nitrophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450 respectively) and low aniline hydroxylase activity (0.03 nmol of p-aminophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450). The residual amount of emulgen 911 is similar in purified P 450 A_2NI and P 450 B_2PB and cannot explain the differences observed in monooxygenase activities. These results confirm our hypothesis, but purified forms could have been more or less inactivated during purification procedure. Consequently, we have performed inhibition experiments in microsomes with antibodies.

The complete inhibition of aniline hydroxylase activity by the anti-A_2NI antibody, irrespective of the type of microsomes confirms that this activity is mainly supported by the cytochrome P 450 A_2NI (Fig. 2A). It indicates too that this P 450 form is present in similar amounts in microsomes from control and phenobarbital-treated rats. Aniline hydroxylase is not affected by the anti P 450 B_2PB antibody, excluding the involvement of the cytochrome P 450 B_2PB in this activity (Fig. 2B). The faint contamination of the anti P 450 A_2NI antibody by anti P 450 B did not modify this result because P 450 B forms are very slightly active on aniline hydroxylase activity. The inhibition patterns of other monooxygenase activities by anti P 450 A_2NI antibody could not be tested because of limited amounts of the antibody.



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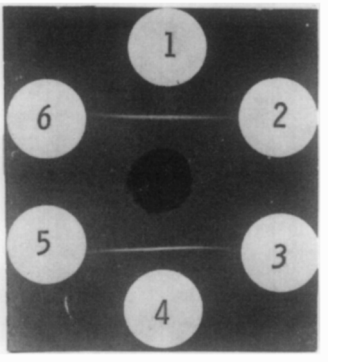

B

Fig. 1A : Ouchterlony double-diffusion analysis

Center well : 0.28 mg anti P 450 A_2NI antibody
Peripheral wells : 0.05 nmol cytochrome P 450 (1 : P 450 A_2NI ; 2 : P 450 B NI ; 3 : P 450 B_2PB ; 4,5,6 : Hemoglobin).

Fig. 1B : Ouchterlony double-diffusion analysis

Center well : 0.046 mg of P 450 B_2PB-IgG antibody
Peripheral wells : 0.04 nmol.cytochrome P 450 (1 : P 450 B_2PB, 2 : P 450 A_2PB, 4 : P 450 B_2PB, 6 : P 450 A_2NI) ; 0.14 nmol. cytochrome P 450 (3 : P 450 A_2PB, 5 : P 450 A_2NI).

The inhibition of benzphetamine N demethylase activity by the anti-P 450 B_2PB antibody (Fig. 3A) shows that the cytochrome P 450 B_2PB is the main form responsible for this activity in microsomes from phenobarbital-treated rats, while in control microsomes, other forms of cytochrome P 450 are able to metabolize benzphetamine. The slight inhibition of the enzymatic activity in control microsomes suggests the presence in these microsomes of a little amount of cytochrome P 450 B_2PB. This result agrees with previous reports from Thomas et al. (23) who have found 2 percent of the form of cytochrome P 450 induced by phenobarbital treatment (called P 450 b) in control microsomes. Moreover, the phenobarbital-induced P 450 has been localized by immunochemistry in control rat liver (24). Thomas et al. (25) have observed different inhibition pattern by the antibody : no difference appeared between control and phenobarbital-treated animals. We have no explanation for this discrepancy.

The inhibition of 4-nitroanisole O demethylase activity by anti P 450 B_2PB antibody looks like the one observed with benzphetamine N demethylase, but the inhibition observed in microsomes from phenobarbital-treated

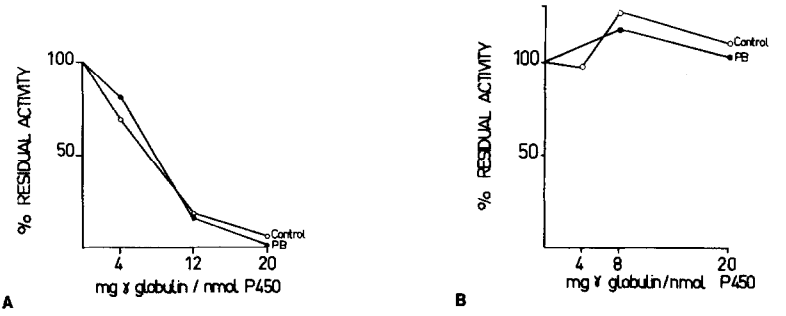

Fig. 2A : Effect of varying amount of A_2NI-IgG antibody on aniline hydroxylation catalyzed by liver microsomes from control and PB-treated rats. The turnover numbers for PB and NI microsomes were 0.26 and 0.45 nmol of p-aminophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450, respectively.

Fig. 2B : Effect of varying amount of B_2PB-IgG antibody on aniline hydroxylation, catalyzed by liver microsomes from control and PB-treated rats. The turnover numbers for PB and NI microsomes were 0.30 and 0.53 nmol of p-aminophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450, respectively.

rats is weaker (Fig. 3B). This indicates that the cytochrome P 450 B_2PB is responsible for an important part of this activity in microsomes from phenobarbital-treated rats, but that there are other forms able to metabolize 4-nitroanisole. The anti P 450 B_2PB antibody inhibits 4-nitroanisole-O-demethylase activity to a lesser extent than benzphetamine N-demethylase activity, which indicates that cytochrome P 450 B_2PB is more specific towards the lat-

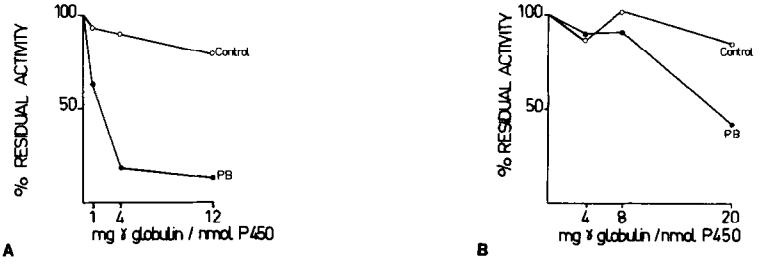

Fig. 3A : Effect of varying amount of B_2PB-IgG antibody on benzphetamine N demethylation catalyzed by liver microsomes from control and PB-treated rats. The turnover numbers for PB and NI microsomes were 7 and 2.3 nmol of formaldehyde formed x min^{-1} x $nmol^{-1}$ cytochrome P 450, respectively.

Fig. 3B : Effect of varying amount of B_2PB-IgG antibody on 4-nitroanisole O demethylation catalyzed by liver microsomes from control and PB-treated rats. The turnover numbers for PB and NI microsomes were 2 and 1.2 nmol of p-nitrophenol formed x min^{-1} x $nmol^{-1}$ cytochrome P 450, respectively.

ter activity. In control microsomes, it is confirmed that the cytochrome P 450 B_2PB exists and that there are other forms responsible for this activity.

In conclusion, we have clearly shown that in liver microsomes from untreated rats, the cytochrome P 450 A_2NI is the major form responsible for aniline hydroxylase activity. In these microsomes, there exists other cytochrome P 450_s and beyond them a little quantity of P 450 B_2PB. In microsomes from phenobarbital-treated animals, P 450 B_2PB is the major form mainly responsible for benzphetamine- and 4-nitroanisole-demethylase activities. In these microsomes, the cytochrome P 450 A_2NI is present and responsible for almost all aniline hydroxylase activity.

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