Biochem. J. (1992) 288, 483–488 (Printed in Great Britain)

Characterization of a phenobarbital-inducible cytochrome *P*-450, NADPH–cytochrome *P*-450 reductase and reconstituted cytochrome *P*-450 mono-oxygenase system from rat brain

Evidence for constitutive presence in rat and human brain

Hindupur K. ANANDATHEERTHAVARADA,* Michael R. BOYD† and Vijayalakshmi RAVINDRANATH*‡
* Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore-560029, India, and † Laboratory of Drug Discovery Reseach and Development, National Cancer Institute, National Institutes of Health, Frederick, MD 21702-1201, U.S.A.

Cytochrome *P*-450 was purified to apparent homogeneity from the brain microsomes of phenobarbital-treated rats. The specific content of the purified *P*-450 was 12.7 nmol/mg of protein. NADPH–cytochrome *P*-450 reductase (reductase) was also purified to apparent homogeneity from brain microsomes. The specific content was 34.7 μmol of cytochrome *c* reduced/min per mg of protein. The reduced carbon monoxide spectrum of purified *P*-450 exhibited a peak at 450 nm. Both the *P*-450 and the reductase moved as single bands on SDS/PAGE. The molecular masses of the purified *P*-450 and the reductase were determined to be 53.3 and 72.0 kDa respectively. The purified brain *P*-450 cross-reacted with antibodies to rat liver *P*-450IIB1/IIB2 when examined by Western immunoblotting, but no immunological similarity was observed with rat liver *P*-450IA1/IA2 or *P*-450IIE1. Purified rat brain reductase cross-reacted with antibodies to rat liver reductase. Further, immunoblot experiments with untreated rat and human brain microsomes using antisera to the purified rat brain *P*-450 and reductase indicated that these forms of *P*-450 and NADPH–cytochrome *P*-450 reductase exist constitutively in rat and human brain. Purified rat brain *P*-450 was reconstituted with purified NADPH–cytochrome *P*-450 reductase, deoxycholate and dilauroyl glyceryl 3-phosphocholine. NADPH-dependent *N*-demethylation of aminopyrine and morphine was observed in the reconstituted system. The catalytic-centre activities were 80.25 and 38.2 nmol of formaldehyde formed/min per nmol of *P*-450 respectively. The reconstituted system had a comparatively lower catalytic-centre activity for 7-ethoxycoumarin *O*-de-ethylase (10.5 nmol of product formed/min per nmol of *P*-450).

INTRODUCTION

Cytochromes *P*-450, a family of haem-containing enzymes, exist in multiple forms and are involved in both detoxification and bioactivation of xenobiotics [1]. The liver is the major organ involved in *P*-450-mediated xenobiotic metabolism, and multiple forms of hepatic *P*-450 have been purified from experimental animals and humans [2,3]. Cytochrome *P*-450 has also been purified from extrahepatic organs, e.g. kidney [4], lung [5,6] and ventral prostate [7]. The brain is the target for a number of toxic compounds and psychoactive drugs [8], and a role for environmental toxins in the aetiopathogenesis of certain neurodegenerative disorders has been suggested [9]. Studies on brain cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase have revealed that these proteins could have diverse functions in the brain. Thus evidence has been presented indicating that the $DM_1/\sigma 1$ binding site could be a member of the cytochrome *P*-450 family [10], and that NADPH–cytochrome *P*-450 reductase shares partial sequence similarity with NADPH diapharose [11] and nitric oxide synthase [12]. In light of the above, further elucidation of the components of the *P*-450 system in the brain is of interest. The purification and characterization of various forms of *P*-450 and NADPH–cytochrome *P*-450 reductase from the brain may help identify the diverse roles of these proteins in this organ.

Previous studies from our laboratory have demonstrated the presence of substantial amounts of *P*-450 in mouse brain [13], rat brain [14] and human autopsy brain [15]. The co-localization of *P*-450 and reductase in the neuronal soma has also been demonstrated [16]. The cytochrome *P*-450 content in the rat brain is typically one-tenth of the hepatic levels, and it exists in multiple forms which are selectively induced by phenobarbital and 3-methylcholanthrene [14]. Pretreatment with phenobarbital induces the cerebral *P*-450 (188% induction) and reductase (270% induction) activities, and this is accompanied by selective induction of the mono-oxygenase (namely, aminopyrene *N*-demethylase and morphine *N*-demethylase) activities known to be mediated by *P*-450IIB1/IIB2 [14]. Chronic administration of phenytoin is also known to induce the cytochrome *P*-450 level in mouse brain [17]. We report here the isolation and characterization of cytochrome *P*-450 and NADPH–cytochrome *P*-450 reductase from the brain microsomes of rats treated with phenobarbital, the reconstitution of mono-oxygenase activity using these purified components and immunological evidence for the constitutive presence of this *P*-450 system in both rat and human brain.

MATERIALS AND METHODS

Materials

Dithiothreitol, phenylmethanesulphonyl fluoride, Tris, sodium cholate, DEAE-Sephacel, Sepharose 4B, Lubrol PX, Triton N-101, 2′,5′-ADP–agarose, dilauroyl glyceryl-3-phosphocholine, sodium deoxycholate, aminopyrine and 7-ethoxycoumarin were purchased from Sigma Chemical Co. All other chemicals were purchased locally and were of analytical grade.

Treatment of animals

One hundred male adult Wistar rats (each weighing 200–220 g) were given phenobarbital (0.025%, w/v) in their drinking water

‡ To whom correspondence should be addressed.

for 70 days. Thereafter, the animals were administered phenobarbital daily (80 mg/kg body weight, intraperitoneally) for 10 days. The animals were killed 24 h after the last dose.

Preparation of microsomes

Microsomes from brains of phenobarbital-treated rats were prepared as described elsewhere [18]. The microsomes were resuspended in 0.1 M-potassium phosphate buffer (pH 7.25) containing 30% (v/v) glycerol, 1 mM-EDTA, 1 mM-dithiothreitol, 22 μM butylated hydroxytoluene and 0.1 mM-phenylmethanesulphonyl fluoride (buffer A). The buffer had been previously bubbled through with nitrogen gas. The microsomes were divided into portions and stored in liquid nitrogen. Microsomes also were prepared from 10 untreated male rat brains as described above.

Cortical microsomes were prepared from human autopsy brain. The deceased, aged 35 years, was a victim of traffic accident with no known neurological disorders. The patient did not receive any anticonvulsant medication prior to death. The interval between death and autopsy was 3 h.

Cytochrome *P*-450 was estimated from the reduced carbon monoxide spectrum [19]; NADPH–cytochrome *c* reductase activity was measured as described [20] during the various stages of purification. Protein was estimated by a dye-binding method [21] and the results were compared with those obtained using the method of Lowry *et al.* [22].

Purification of *P*-450 and NADPH–*P*-450 reductase from brains of phenobarbital-treated rats

All procedures were carried out at 4 °C and all buffers used for purification were bubbled with nitrogen gas prior to use. Microsomes were diluted to final concentration of 2 mg/ml in buffer A. To this was added 20% (w/v) sodium cholate dropwise for 15 min to a final concentration of 0.6% (w/v) with gentle stirring. After stirring for an additional 90 min at 4 °C, the solution was centrifuged at 100000 *g* for 1 h and the supernatant was collected.

The supernatant (200 mg of microsomal protein) was loaded on to a column (2.5 cm × 35 cm) packed with octylamino Sepharose 4B, which had been previously equilibrated with 1 litre of 0.1 M-potassium phosphate buffer (pH 7.25) containing 1 mM-EDTA, 1 mM-dithiothreitol, 20% (v/v) glycerol and 0.7% (w/v) sodium cholate. The flow rate was adjusted to 0.5 ml/min. The column was washed with the above buffer (500 ml) containing 0.46% (w/v) sodium cholate. The flow rate was 1 ml/min. Cytochrome *P*-450 was eluted as a sharp peak with 0.1 M-potasium phosphate buffer (pH 7.25) containing 1 mM-EDTA, 1 mM-dithiothreitol, 20% (v/v) glycerol, 0.3% (w/v) sodium cholate and 0.08% (v/v) Triton N-101. The absorbance of the eluate was monitored at 417 nm.

After the elution of the *P*-450, the reductase was eluted from the octylamino Sepharose 4B column with 0.1 M-potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1 mM-EDTA, 1 mM-dithiothreitol and 0.37% (w/v) sodium deoxycholate. NADPH–cytochrome *c* reductase activity was monitored in the eluted fractions.

The cytochrome *P*-450-containing fractions from the octylamino Sepharose 4B column were pooled and concentrated to half the original volume using an Amicon PM-30 membrane, and dialysed overnight against 10 vol. of a solution containing 20% (v/v) glycerol, 0.1 mM-dithiothreitol and 0.1 mM-EDTA. The cytochrome *P*-450-containing fractions were dialysed further against 10 mM-potassium phosphate buffer (pH 7.7) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 20% (v/v) glycerol, 0.2% (w/v) sodium cholate and 0.1% (v/v) Lubrol PX.

Following dialysis, the non-diffusible material was applied to a DEAE-Sephacel column (2.5 cm × 30 cm) which had been previously equilibrated with 1 litre of 10 mM-potassium phosphate buffer (pH 7.7) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 20% (v/v) glycerol, 0.2% (w/v) sodium cholate and 0.1% (v/v) Lubrol PX, at a flow rate of 1 ml/min. The major amount of *P*-450 was eluted with the above buffer. Two small peaks with very low *P*-450 concentrations were eluted with the above buffer containing NaCl (200 and 300 mM respectively). The major fraction that was eluted with the equilibration buffer was passed through a column (1.5 cm × 25 cm) packed with XAD-2 beads, at a flow rate of 0.25 ml/min. The eluant was concentrated using an Amicon PM-30 membrane and dialysed overnight against 20 vol. of 10 mM-Tris/acetate buffer (pH 7.4) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol and 20% (v/v) glycerol. The non-diffusible material containing *P*-450 was divided into portions and stored at 70 °C until further analysis.

The NADPH–cytochrome *P*-450 reductase-containing fractions which eluted from the octylamino Sepharose-4B column were pooled and loaded directly (at a flow rate of 0.25 ml/min) on to a small column of 2′,5′-ADP–agarose, which was equilibrated with 0.25 M-potassium phosphate buffer (pH 7.25) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 20% (v/v) glycerol, 2 μM-FMN and 0.1% (v/v) Lubrol PX. The column was washed with 30 mM-potassium phosphate buffer (pH 7.7) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 20% (v/v) glycerol, 2 μM-FMN and 0.1% (w/v) sodium deoxycholate. The NADPH–cytochrome *c* reductase activity was eluted as a sharp peak with the above buffer containing 2′-AMP (5 mM). The fractions were pooled, concentrated and dialysed against 5 vol. of 10 mM-Tris/acetate buffer (pH 7.4) containing 0.1 mM-EDTA, 0.1 mM-dithiothreitol and 20% (v/v) glycerol. The non-diffusible material containing the reductase was divided into portions and stored at −70 °C.

Immunochemical studies

Antisera to purified rat brain *P*-450 and reductase were raised in male New Zealand White rabbits [4]. Antisera to purified rat liver *P*-450IIB1/IIB2, *P*-450IA1/IA2 and reductase were raised in rabbits and characterized as described elsewhere [13,14,16]. Antiserum to rat liver *P*-450IIE1 was a gift from Dr. B. J. Song, NIAAA, Rockville, MD, U.S.A., and this antiserum was characterized as described [23].

The purified brain proteins (*P*-450 and reductase) were subjected to SDS/PAGE using 10% acrylamide (see Fig. 1*a*) or a gradient of 3–10% acrylamide (see Fig. 1*b*). The gels were stained with Coomassie Blue [24]. The molecular masses of purified brain *P*-450 and reductase were determined using standard molecular mass markers. The purified proteins and microsomes from untreated and phenobarbital-rat liver and brain and human autopsy brain were also subjected to SDS/PAGE and then transferred on to nitrocellulose membranes for examination by immunoblot [25]. After the incubation with antisera, the blots were immunostained as described [13].

Reconstitution of purified *P*-450 and reductase

The catalytic activity of purified rat brain *P*-450 in a reconstituted system containing purified rat brain reductase and dilauroyl glyceryl-3-phosphocholine was investigated as described [5] with certain modifications as detailed below. The system was reconstituted in 50 mM-Tris buffer (pH 7.4), containing 20 mM-$MgCl_2$, 40 μM-dilauroyl glyceryl-3-phosphocholine, 0.18 μM-sodium deoxycholate, purified rat brain reductase (3.5 μg), 1 mM substrate and purified rat brain *P*-450 (30–60 pmol). The reaction was initiated by addition of NADPH to a final concentration of 1 mM. The total volume was 1 ml. The formaldehyde formed during the *N*-demethylation of amino-

Table 1. Specific content of rat brain *P*-450 at various stages of purification

Fraction	Total protein (mg)	Specific activity (nmol of *P*-450/mg)	Total activity (nmol of *P*-450)	Overall yield (%)	Purification (fold)
Microsomes	400	0.22	88.8	100	–
Solubilized microsomes	200	0.38	75.4	85	1.6
Elution from octylamino Sepharose 4B	33.8	1.7	56.0	63	7.5
Elution from DEAE-Sephacel (10 mM-potassium phosphate)	0.7	12.7	9.4	10.6	57.4

Table 2. Specific activity of rat brain NADPH–*P*-450 reductase at various stages of purification

Reductase activity is expressed as nmol of cytochrome *c* reduced/min per mg of protein. Reductase activity was determined in the presence of 0.6% (w/v) sodium cholate and 2 μM-FMN. In the absence of sodium cholate and FMN, the reductase activity in microsomes was 55.2 nmol of cytochrome *c* reduced/min per mg of protein.

Fraction	Total protein (mg)	Specific activity (nmol/min per mg)	Total activity (nmol/min)	Overall yield (%)	Purification (fold)
Microsomes	400	162	68800	100	–
Solubilized microsomes	200	247	49400	76	1.5
Elution from octylamino Sepharose 4B	20	2160	43100	67	13.0
Elution from 2′5′-ADP–agarose	0.97	34700	33700	52	214

pyrine or morphine was determined using NASH reagent [26]. The assay of 7-ethoxycoumarin *O*-de-ethylase activity in the reconstituted system was carried out as described [27].

RESULTS

Purification of *P*-450 from brain microsomes of phenobarbital-treated rats was carried out using a combination of octylamino Sepharose 4B and DEAE-Sephacel chromatography as described earlier [5]. The summary of the purification of *P*-450 is given in Table 1. A total of 85% of the brain microsomal *P*-450 was recovered on solubilization of microsomes with sodium cholate. The chromatography of solubilized microsomes on octylamino Sepharose 4B resulted in an increase in the specific content of *P*-450 by 44%. The recovery from this step was 74%. The eluant from the octylamino Sepharose 4B column was dialysed and applied to a DEAE-Sephacel column, and was eluted in low-ionic-strength buffer (10 mM-potassium phosphate buffer). However, a small quantity of *P*-450 was eluted as two small peaks with 200 and 300 mM-NaCl. The specific content of *P*-450 in these fractions eluting in 10 mM-phosphate buffer was high (12.74 nmol of *P*-450/mg of protein). The overall recovery of *P*-450 was 10.6%.

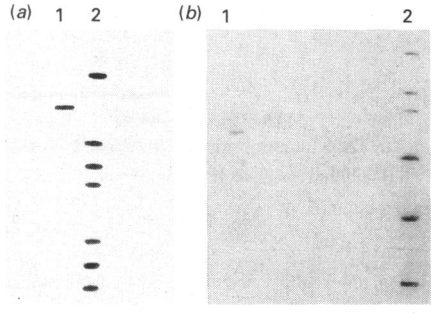

Fig. 1. SDS/PAGE of purified brain *P*-450 (*a*) and NADPH–*P*-450 reductase (*b*)

(*a*) Lane 1, purified brain *P*-450 (5 μg). Lane 2, standard molecular mass markers (top to bottom): BSA (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.2 kDa). (*b*) Lane 1, purified reductase (5 μg). Lane 2, standard molecular mass markers (top to bottom): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa).

The results of reductase purification from phenobarbital-treated rat brain are shown in Table 2. Rat brain reductase was separated from *P*-450 on an octylamino Sepharose 4B column and eluted with buffer containing sodium deoxycholate; the recovery was 87% of the enzyme activity present in solubilized microsomes. Affinity chromatography on 2′,5′-ADP–agarose resulted in the separation of purified brain reductase. The enzyme was purified 214-fold and the specific activity was 34.7 μmol of cytochrome *c* reduced/min per mg of protein.

SDS/PAGE of the purified *P*-450 and reductase is shown in Figs. 1(*a*) and 1(*b*). The proteins appear as single bands on the gel. The molecular mass of brain *P*-450 was determined to be 53.3 kDa and that of the reductase was determined to be 72.0 kDa.

The reduced carbon monoxide spectrum of purified *P*-450 exhibited a single peak at 450 nm (Fig. 2). The absence of any peak at 420 nm indicated that purified *P*-450 was devoid of the denatured form of *P*-450, namely cytochrome *P*-420.

The purified *P*-450 efficiently catalysed the *N*-demethylation of aminopyrine in a reconstituted system containing reductase, deoxycholate and dilauroyl glyceryl-3-phosphocholine (Table 3). The catalytic-centre activity for aminopyrine *N*-demethylation was 80.3 nmol of formaldehyde formed/min per nmol of *P*-450. The presence of reductase was essential for *P*-450 catalytic activity. The absence of deoxycholate or dilauroyl glyceryl-3-phosphocholine caused a substantial decrease in the turnover number (Table 3).

The capability of the reconstituted *P*-450 system to metabolize various other substrates was also examined (Table 4). Among the substrates tested, the highest catalytic-centre activity was observed for aminopyrine, followed by morphine. The de-ethylation of 7-ethoxycoumarin was catalysed to a lesser extent.

Antisera raised against purified rat brain *P*-450 identified a

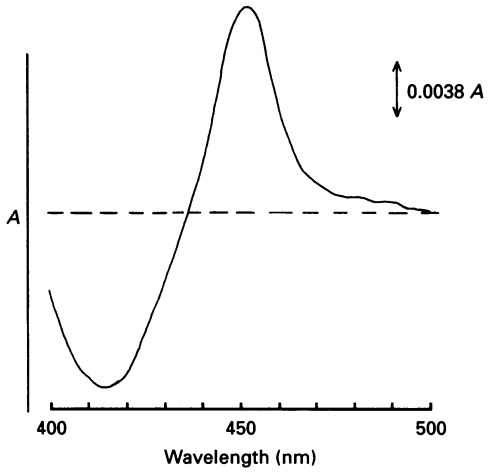

Fig. 2. Reduced carbon monoxide spectrum of purified rat brain *P*-450

The concentration of protein was 15 μg/ml. The broken line indicates the baseline.

Table 3. Aminopyrine *N*-demethylase activity in a reconstituted system containing purified brain *P*-450 and NADPH–*P*-450 reductase

Reconstitution was carried out in 50 nM-Tris buffer (pH 7.4) containing $MgCl_2$ (20 mM), sodium deoxycholate (0.18 μM), purified rat brain reductase (3.5 μg), dilauroyl glyceryl-3-phosphocholine (40 μM), purified rat brain *P*-450 (30 or 60 pmol), aminopyrine (1 mM) and NADPH (1 mM) in a total volume of 1 ml. The formaldehyde formed was measured as described in the Materials and methods section. The assays were performed in duplicate. ND indicates that activity was not detectable.

System	Turnover number (nmol of formaldehyde formed/min per nmol of *P*-450)
Complete system	80.3
No deoxycholate	32.5
No dilauroyl glyceryl-3-phosphocholine	24.0
No reductase	ND
No *P*-450	ND
No aminopyrine	ND

Table 4. Activity of purified phenobarbital-treated rat brain microsomal *P*-450 towards various substrates in a reconstituted system

Assays were carried out as described in the Materials and methods section and were performed in duplicate.

Substrates (1 mM)	Activity
Aminopyrine	80.3*
Morphine	38.2*
7-Ethoxycoumarin	10.5†

* nmol of formaldehyde formed/min per nmol of *P*-450.
† nmol of 7-hydroxycoumarin formed/min per nmol of *P*-450.

single protein band in untreated rat brain microsomes and human brain microsomes, when examined by Western immunoblotting (Fig. 3). These bands co-migrated with purified rat brain *P*-450.

A typical Western immunoblot of purified rat brain *P*-450 incubated with antisera to rat liver *P*-450IA1/IA2, *P*-450IIB1/IIB2 or *P*-450IIE1 exhibited cross-reactivity only with antisera to rat liver *P*-450IIB1/IIB2 (Fig. 3).

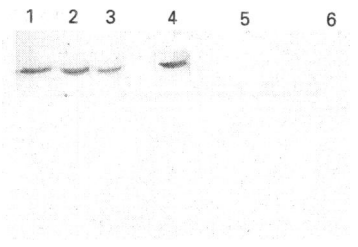

Fig. 3. Immunoblots of purified brain *P*-450 and untreated rat and human brain microsomes following immunostaining with antisera to purified rat brain *P*-450 (lanes 1, 2 and 3), and immunoblot of purified brain *P*-450 following immunostaining with antisera to various forms of rat liver *P*-450 (lanes 4, 5 and 6)

Amounts loaded: purified rat brain *P*-450; 2 μg (lane 1), untreated rat brain microsomes, 30 μg (lane 2); human brain cortical microsomes, 40 μg (lane 3). Immunoblotting of purified brain *P*-450 (2 μg/lane) is shown following immunostaining with antisera to rat liver *P*-450IIB1/IIB2 (lane 4), rat liver *P*-450IA1/IA2 (lane 5) and rat liver *P*-450IIE1 (lane 6).

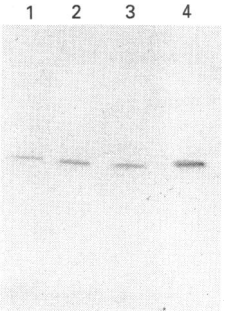

Fig. 4. Immunoblots of brain microsomes from control (lanes 1 and 3) and phenobarbital-treated rats (lanes 2 and 4) following immunostaining with antiserum to purified rat brain *P*-450

The amount of brain microsomal protein contents in lanes 1 and 2 was 50 μg, and that in lanes 3 and 4 was 75 μg.

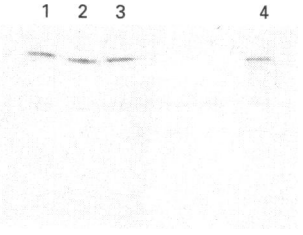

Fig. 5. Immunoblots of purified brain NADPH–*P*-450 reductase and untreated rat and human brain microsomes, following staining with antiserum to purified rat brain reductase

Lane 1, purified rat brain reductase (1 μg/lane); lane 2, untreated rat brain microsomes (30 μg/lane); lane 3, human brain cortical microsomes (40 μg/lane); lane 4, immunoblot of purified brain reductase (2 μg/lane) immunostained with antiserum to rat liver reductase.

Immunoblot experiments with control and phenobarbital-treated rat brain microsomes using antisera to purified rat brain *P*-450 revealed the presence of a single band in each case. However, the band was substantially more intense in the lane containing phenobarbital-treated rat brain microsomes compared with control rat brain microsomes (Fig. 4).

Antisera raised against purified rat brain NADPH–*P*-450 reductase identified a single protein band in untreated rat brain microsomes and human brain microsomes when examined by Western immunoblotting (Fig. 5). These bands co-migrated with purified rat brain reductase. A typical immunoblot of purified rat brain reductase incubated with antiserum to rat liver reductase is shown in Fig. 5. The immunoblot revealed a single protein band, indicating cross-reactivity between purified rat brain reductase and antiserum to rat liver reductase.

DISCUSSION

Evidence for the presence of *P*-450 in rat brain microsomes was first presented more than a decade ago [28,29], but to date the *P*-450 from brain microsomes has not been purified to homogeneity. Partial purification of rat brain microsomal *P*-450 had been reported previously [30]; the specific content of the partially purified brain *P*-450 was 0.18 nmol of *P*-450/mg of protein, which was about one-hundreth the specific content of purified hepatic *P*-450. The partial purification of brain *P*-450 using whole brain homogenate had also been reported by Warner *et al.* [31]; the specific content of *P*-450 was 1–3 nmol of *P*-450/mg of protein. Since the mitochondrial *P*-450 content in brain was reported to be relatively high [32], the specific content of the partially purified *P*-450 from whole brain homogenate conceivably could have included contributions from both microsomal and mitochondrial *P*-450s. The purification of the brain microsomal *P*-450 offered us a particular challenge, since this organ contains very low amounts of *P*-450. Further, brain microsomal *P*-450 had been reported to be very unstable during the purification process [30]. Similarly, the purification of *P*-450 from other extrahepatic organs had been reported to be especially difficult due to the relatively low amounts of *P*-450 present. For example, the specific content of the purified *P*-450 from rat ventral prostate was reported to be 8–10 nmol of *P*-450/mg of protein [7] and the specific content of *P*-450 purified from rat kidney was reported to be 10–12.6 nmol of *P*-450/mg of protein [4].

Isolation of brain microsomes in the presence of glycerol and dithiothreitol [8] helps preserve the integrity of *P*-450, and higher levels of *P*-450 (0.08 nmol/mg of protein) were detectable in untreated rat brain compared with earlier reports (0.03 nmol/mg of protein). Following prolonged treatment with phenobarbital the *P*-450 levels in brain are induced, resulting in a higher *P*-450 content (0.22 nmol/mg of protein; 250% induction). Therefore phenobarbital-induced rat brain microsomes were used for purification of this form of *P*-450 from rat brain.

In the present study the purified brain *P*-450 had a specific content of 12.74 nmol of *P*-450/mg of protein. The *P*-450 from phenobarbital-treated rat brain was eluted with low-ionic-strength buffer (10 mM potassium phosphate) from a DEAE-Sephacel column. Similar behaviour has been reported for rabbit lung *P*-450 [5] and the *P*-450 from *Drosophila melanogaster* [33]. SDS/PAGE of purified rat brain *P*-450 revealed the apparently homogeneous nature of the preparation (Fig. 1*a*). The absorption maximum of the reduced carbon monoxide spectrum of purified brain *P*-450 was indeed observed at 450 nm.

The NADPH–*P*-450 reductase was purified to apparent homogeneity from the phenobarbital-treated rat brain microsomes. SDS/PAGE analysis of the purified rat brain reductase revealed that the final preparation was apparently homogeneous (Fig. 1*b*). NADPH–cytochrome *c* reductase activity detectable in microsomes increased significantly in the presence of sodium cholate and FMN. The specific activity of the purified brain reductase was lower than that of a similar preparation from rat liver (40 compared with 70 μmol of cytochrome *c* reduced/mg of protein [34]).

In the reconstituted system, the purified rat brain *P*-450 exhibited a high catalytic-centre activity for the metabolism of aminopyrine (80.3 nmol of product formed/min per nmol of *P*-450). The purified *P*-450 from phenobarbital-treated rat liver was also reported to have a high catalytic-centre activity for the metabolism of aminopyrine (85 nmol of product formed/min per nmol of *P*-450) and a low catalytic-centre activity for the metabolism of 7-ethoxycoumarin (9 nmol of product formed/min per nmol of *P*-450) [2]. We observed similar results with the reconstituted system from brain; the catalytic-centre activity for 7-ethoxycoumarin *O*-de-ethylase was low (10.5 nmol of product formed/min per nmol of *P*-450) (Table 4).

The purified rat brain *P*-450 exhibited immunological cross-reactivity with antiserum to rat liver *P*-450IIB1/IIB2, but no cross-reactivity was observed with antisera to *P*-450IA1/IA2, and *P*-450IIE1. The purified rat brain reductase also exhibited cross-reactivity with antiserum to rat liver reductase, indicating immunological similarity between purified brain reductase and rat liver reductase.

In summary, a cytochrome *P*-450 and an NADPH–cytochrome *P*-450 reductase have been purified successfully from brains of phenobarbital-treated rats and reconstituted mono-oxygenase activities have been demonstrated. Immunoblot experiments with untreated rat and human brain microsomes, using antisera to the purified rat brain *P*-450 and reductase, indicated that this form of *P*-450 and reductase exist constitutively in rat and human brains. The characterization of the phenobarbital-inducible form of *P*-450 in brain is of particular interest in view of the extensive use of phenobarbital as an anticonvulsant agent.

We thank Dr. B. J. Song for providing antibodies to rat liver cytochrome *P*-450IIE1, Dr. S. K. Shankar for providing human autopsy brain tissue, and Shripad Bhagwat for performing some of the immunoblot experiments. This work was supported through the U.S.–India Fund for Cultural, Educational and Scientific Co-operation.

REFERENCES

1. Oritz de Montellano, P. R. (1986) Cytochrome *P*-450: Structure, Mechanism and Biochemistry, Plenum Press, New York
2. Guengerich, F. P. (1987) in Mammalian Cytochromes *P*-450 (Guengerich, F. P., ed.), vol. I, pp. 1–54, CRC Press, Boca Raton
3. Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1989) DNA **8**, 1–13
4. Imaoka, S., Nagasimha, K. & Funae, Y. (1990) Arch. Biochem. Biophys. **276**, 473–480
5. Guengerich, F. P. (1977) Mol. Pharmacol. **13**, 911–923
6. Slaughter, S. R., Wolf, C. R., Marciniszyn, J. P. & Philpot, R. M. (1981) J. Biol. Chem. **256**, 2499–2503
7. Sundin, M., Warner, M., Haaparanta, T. & Gustafsson, J. A. (1987) J. Biol. Chem. **262**, 12293–12297
8. Mensil, M., Testa, B. & Jenner, P. (1984) Adv. Drug Res. **13**, 95–207
9. Calne, D. B., McGeer, E., Eisen, A. & Spencer, P. (1986) Lancet **ii**, 1067–1070
10. Klein, M., Canoll, P. D. & Musacchio, J. M. (1991) Life Sci. **48**, 543–550
11. Kemp, M. C., Knoneu, D. R., Sutton, A. & Roberts, P. J. (1988) Biochem. Pharmacol. **37**, 3063–3070
12. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) Nature (London) **351**, 714–718
13. Ravindranath, V. & Anandatheerthavarada, H. K. (1989) Biochem. J. **261**, 769–773
14. Anandatheerthavarada, H. K., Shankar, S. K. & Ravindranath, V. (1990) Brain Res. **536**, 339–343
15. Ravindranath, V., Anandatheerthavarada, H. K. & Shankar, S. K. (1989) Brain Res. **496**, 331–335

16. Ravindranath, V., Anandatheerthavarada, H. K. & Shankar, S. K. (1990) Biochem. Pharmacol. **39**, 1013–1018
17. Volk, B., Amelizad, Z., Anagnostopoulos, J., Knoth, R. & Oesch, F. (1988) Neurosci. Lett. **84**, 219–224
18. Ravindranath, V. & Anandatheerthavarada, H. K. (1990) Anal. Biochem. **187**, 310–313
19. Matsubara, R., Koike, M., Tochino, Y. & Sugeno, K. (1976) Anal. Biochem. **76**, 596–603
20. Phillips, A. H. & Langdon, R. G. (1962) J. Biol. Chem. **237**, 2652–2660
21. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
23. Song, B. J., Gelboin, H. V., Park, S. S., Yang, C. S. & Gonzalez, F. J. (1986) J. Biol. Chem. **261**, 16689–16697
24. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25. Towbin, H., Staehelin, R. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
26. Werringloer, J. (1978) Methods Enzymol. **52**, 297–302
27. Greenlee, W. F. & Poland, A. (1978) J. Pharmacol. Exp. Ther. **205**, 596–605
28. Sasame, H. A., Ames, M. M. & Nelson, S. D. (1977) Biochem. Biophys. Res. Commun. **78**, 919–926
29. Marietta, M. P., Vessel, E. S., Hartmann, R. D., Weisz, J. & Dvorchik, B. H. (1979) J. Pharmacol. Exp. Ther. **208**, 271–279
30. Sugita, O., Miyairi, S., Sassa, S. & Kappas, A. (1987) Biochem. Biophys. Res. Commun. **147**, 1245–1250
31. Warner, M., Kohler, C., Hansson, T. & Gustafsson, J. A. (1988) J. Neurochem. **50**, 1057–1065
32. Walther, B., Ghersi-Egea, J. F., Minn, A. & Siest, G. (1986) Brain Res. **375**, 338–344
33. Sundseth, S. S., Nix, C. E. & Waters, L. C. (1990) Biochem. J. **265**, 213–217
34. Guengerich, F. P. (1984) in Principles and Methods of Toxicology (Hayes, A. W., ed.), pp. 609–634, Raven Press, New York

Received 29 May 1992; accepted 23 June 1992