Purification and characterization of an acetone-inducible cytochrome *P*-450 from hamster liver microsomes

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A form of cytochrome *P*-450 has been purified to electrophoretic homogeneity from the hepatic microsomes of Syrian golden hamsters treated with acetone. This *P*-450 form, designated ha *P*-450_j, had an M_r of ~55000, bound dimethyl sulphoxide and exhibited a CO-reduced absorbance maximum at 451 nm. The absolute spectra of its oxidized form indicated that ha *P*-450_j was predominantly in the low-spin state. In a reconstituted system, ha *P*-450_j showed relatively low catalytic activities towards 7-ethoxycoumarin, 7-ethoxyresorufin, aminopyrine, ethylmorphine and benzphetamine, whereas it catalysed the oxidation of aniline, acetone and thiobenzamide with a high catalytic-centre activity. In addition, ha *P*-450_j catalysed at a high rate the high-affinity component of dimethylnitrosamine *N*-demethylase; in contrast, only the low-affinity component of diethylnitrosamine *N*-de-ethylase was efficiently catalysed. The addition of cytochrome *b*₅ to the reconstitution system decreased the K_m value for dimethylnitrosamine *N*-demethylase by a factor of 5 and increased the V_{max} value, and slightly enhanced the other activities. Thiobenzamide and diethyldithiocarbamate were found to be the most effective inhibitors of the ha-*P*-450_j-dependent aniline hydroxylation. Polyclonal antibodies against rat *P*-450, recognized ha *P*-450_j in immunoblots of control and treated hamster liver microsomes. Treatment of hamsters with acetone increased the apparent abundance of ha *P*-450_j in microsomes, whereas phenobarbital and β -naphthoflavone did not induce it. Analysis of *N*-terminal amino acid sequences demonstrated that ha *P*-450_j has a high degree of sequence identity with rat *P*-450_j. All the evidence presented in this study indicates that ha *P*-450_j could represent the hamster orthologue of the previously described CYP2E1(s) of other species.

INTRODUCTION

Cytochromes *P*-450 comprise a superfamily of ubiquitous mono-oxygenases associated with the metabolism of a broad group of structurally unrelated compounds, including drugs, chemical carcinogens, environmental pollutants and endogenous substrates such as steroids, fatty acids and prostaglandins [1,2]. Individual isoenzymes have been grouped according to their responsiveness to various inducers [3], immunochemical relatedness [4,5] and degree of similarity of their respective cDNA sequences [6].

Cytochrome CYP2E1 is a form of *P*-450 belonging to the *CYP2E* gene subfamily which is constitutively present in rodents; it is induced by several xenobiotics [ethanol, acetone, isoniazid and pyrazole (not in mouse [7]) etc.] and by physiological conditions such as fasting and diabetes [8–13]. Its functional properties have generated much interest; this *P*-450 has a remarkable capacity to oxidize ethanol and other primary alcohols [14–16], *p*-nitrophenol [17], acetone [18], aniline [15,19], dimethylnitrosamine (low- K_m) [20] and diethylnitrosamine (low- K_m) [21–23]. It is also able to convert acetaminophen [24] and carbon tetrachloride [25] into highly reactive intermediates.

Orthologous gene products of CYP2E1 have been purified and characterized from rat (also known as *P*-450_j [8,10,13]), rabbit [19] and human [26–28] liver microsomes. The purification of CYP2E1 from hamsters has not been described, although the hamster has become a popular animal model in studies of metabolism and carcinogenesis, since its drug-metabolizing capacity is higher than that of other rodents [29,30].

There are several indications that a CYP2E1 orthologue exists in hamster liver. Treatment of hamsters with typical CYP2E1 inducers such as ethanol [31–35], isoniazid [34] or pyrazole [36] caused an induction of hepatic mono-oxygenase activities associated in rat or rabbit with CYP2E1. Ethanol or pyrazole treatment of hamsters also increased the amount of mRNA which hybridized to a CYP2E1 probe from rat [36]. Moreover, it has been demonstrated that polyclonal antibodies raised against rabbit CYP2E1 [35] or rat CYP2E1 [37] recognize a single protein of M_r about 54000 in Western blots of hamster liver microsomes.

Recently we have reported [38] that treatment of hamsters with acetone, a strong inducer of CYP2E1, caused an increase in many CYP2E1-linked mono-oxygenase activities, but not, unlike in the rat [21,23] or mouse [22], of the high-affinity component of diethylnitrosamine *N*-de-ethylase (DENd) [39].

These findings encouraged us to purify an hepatic *P*-450, termed ha *P*-450_j, from acetone-pretreated adult male hamsters and to determine some of its catalytic and structural features. The results of this study indicated that ha *P*-450_j is similar to the human, rabbit and rat CYP2E1 orthologues, even though some structural and catalytic differences exist between these proteins.

EXPERIMENTAL

Chemicals

Sephacrose 4B was purchased from Pharmacia (Uppsala, Sweden), DEAE-cellulose (DE 52) was from Whatman Biosystem (Clifton, NJ, U.S.A.), and Hypatite C (hydroxyapatite) was

Abbreviations used: PB, phenobarbital; β -NF, β -naphthoflavone; DMN, dimethylnitrosamine; DMNd, dimethylnitrosamine *N*-demethylase; DEN, diethylnitrosamine; DENd, diethylnitrosamine *N*-de-ethylase; AcH, acetone hydroxylase; ECOD, ethoxycoumarin *O*-de-ethylase; AnH, aniline hydroxylase; Ac-microsomes, microsomes from acetone-treated hamster liver; *b*₅, cytochrome *b*₅; *P*-450, cytochrome *P*-450; ha *P*-450_j, acetone-inducible *P*-450 from hamster liver; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline (0.9% NaCl, 10 mM-sodium phosphate, pH 7.2).

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obtained from Clarkson Co. (Williamsport, PA, U.S.A.). Sodium cholate was purchased from Sigma (St. Louis, MO, U.S.A.) and recrystallized twice from ethanol. Emulgen 911 was from KAO Corp. (Tokyo, Japan). Rabbit polyclonal antibodies against rat *P*-450₁ and horseradish peroxidase-coupled goat anti-(rabbit IgG) were purchased from Oxygene (Dallas, TX, U.S.A.) and DAKO (Copenhagen, Denmark) respectively. 7-Ethoxycoumarin was from EGA Chemie (Steinheim, Germany); diethylnitrosamine (DEN), dimethylnitrosamine (DMN), resorufin and acrylamide were obtained from Fluka (Buchs, Switzerland). Enzymes and coenzymes were from Boehringer (Mannheim, Germany). Thiobenzamide was obtained from Aldrich. Benzphetamine and ethylmorphine were supplied by the Upjohn Co. (Kalamazoo, MI, U.S.A.) and Merck (Darmstadt, Germany) respectively. Ethoxyresorufin was synthesized from resorufin by ethylation with ethyl iodide [40]. DEN was distilled before use. Because of the hazardous nature of DEN, its distillation was performed in a restricted area following the safety standard procedures of the International Agency for Research on Cancer. Decontamination of the glassware of DEN was carried out as described by Lunn *et al.* [41]. All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Treatment of animals and isolation of microsomes

Male Syrian golden hamsters (4–5 weeks old; Charles River, Kissleg, Germany) were given acetone (3% in drinking water) *ad libitum* for 10 days. The mean consumption of acetone was 2.1 ± 0.4 g/kg body wt. per day. β -Naphthoflavone (β -NF) was given in corn oil by intraperitoneal injection (40 mg/kg daily for 3 days) and the animals were killed 24 h after the last injection of β -NF. In all cases, after treatments, the animals were killed by CO₂ asphyxia. Livers were homogenized in 0.1 M-Tris/HCl, pH 7.4, buffer containing 1.15% (w/v) KCl and 0.1 mM-phenylmethanesulphonyl fluoride (PMSF).

The homogenate was centrifuged at 10000 *g* for 20 min (2–4 °C). The supernatant fraction was then centrifuged at 100000 *g* for 60 min (2–4 °C); the microsomal pellet obtained was resuspended in 0.1 M-pyrophosphate, pH 7.4, buffer containing 0.1 mM-PMSF and again centrifuged at 100000 *g* for 40 min. The washed microsomes were stored in 0.1 M-potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol and 1 mM-EDTA at –80 °C until use or, in the case of acetone-induced microsomes, resuspended in the solubilization buffer and used for purification.

Purification of acetone-inducible ha *P*-450₁

Ha *P*-450₁ was purified using a modification of the procedure of Palakodety *et al.* [10]. All the purification steps were carried out at 4 °C.

Microsomes obtained from 20 hamster livers were solubilized (2.5 mg of protein/ml) with 0.6% (w/v) recrystallized sodium cholate in 650 ml of 100 mM-potassium phosphate buffer, pH 7.25, containing 1 mM-dithiothreitol (DTT), 0.1 mM-PMSF, 1 mM-EDTA and 20% (v/v) glycerol for 30 min at 4 °C, after which the mixture was centrifuged for 1 h at 100000 *g*. The supernatant, containing about 2000 nmol of cytochrome *P*-450₁, was then applied to an n-octylamine-Sepharose 4B column (2.5 cm × 50 cm) equilibrated with the solubilization buffer. The resin was obtained by coupling 1,8-diamino-octane to cyanogen bromide-activated Sepharose 4B as previously described [42]. After sample application, the column was washed with 1 litre of 20 mM-potassium phosphate buffer (pH 7.25) containing 0.42% (w/v) sodium cholate, 1 mM-DTT, 1 mM-EDTA, 20% (v/v) glycerol and 0.1 mM-PMSF, followed by the same buffer mixture containing 0.33% (w/v) sodium cholate and 0.05% (w/v)

Emulgen 911. The column was then eluted with 1 litre of 100 mM-potassium phosphate buffer (pH 7.25) containing 1 mM-DTT, 1 mM-EDTA, 20% (v/v) glycerol, 0.33% (w/v) sodium cholate and 0.05% (w/v) Emulgen 911. This fraction (fraction I) contained most of the *P*-450₁ (1300 nmol), but not ha *P*-450₁. The purification of acetone-induced *P*-450 was monitored by subjecting each fraction to SDS/PAGE [43], by immunoblot analysis using anti-(rat CYP2E1) and by the dimethyl sulphoxide (DMSO)-binding spectrum (high-affinity component) using the procedure described elsewhere [38].

The n-octylamine-Sepharose 4B column was eluted with the above buffer containing 0.5% (w/v) Emulgen 911. This fraction (fraction II), enriched in acetone-inducible *P*-450, was concentrated by ultrafiltration through an Amicon PM-30 membrane and extensively dialysed against 3 litres of 5 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM-EDTA and 0.1 mM-DTT. After dialysis the concentrations of Emulgen 911 and DTT in the sample were brought to 0.6% (w/v) and 1 mM respectively. The sample was then applied to a Whatman DE 52 anion-exchange column (1.5 cm × 30 cm), previously equilibrated with the dialysis buffer containing 0.6% (w/v) Emulgen 911 and 1 mM-DTT. The column was then washed with 500 ml of the equilibration buffer. The fraction containing ha *P*-450₁, on the basis of SDS/PAGE profiles and DMSO-binding spectra, was recovered in the flow-through.

The fraction obtained was concentrated to 30 ml and dialysed overnight against 2 litres of 5 mM-potassium phosphate buffer, pH 7.7, containing 1 mM-DTT, 1 mM-EDTA and 20% (v/v) glycerol, and loaded on to a Whatman DE 52 anion-exchange column (1.5 cm × 15 cm) equilibrated with the dialysis buffer containing 0.6% (w/v) Emulgen 911. The column was washed with 300 ml of equilibration buffer and unbound *P*-450, which did not bind DMSO, was eluted in the flow-through (fraction A). The ha *P*-450₁-containing fraction (fraction B), which bound DMSO, was eluted from the column by extensive washing with equilibration buffer (500 ml), concentrated and loaded on to a hydroxyapatite column (1.5 cm × 5 cm) equilibrated with 5 mM-potassium phosphate buffer, pH 7.7, containing 0.5% (w/v) sodium cholate, 0.2% (w/v) Emulgen 911, 20% (v/v) glycerol, 1 mM-DTT and 1 mM-EDTA. The column was washed with the equilibration buffer and eluted stepwise with 50, 90 and 250 mM-potassium phosphate buffer, pH 7.7, containing 0.5% (w/v) sodium cholate, 0.2% (w/v) Emulgen 911, 20% (v/v) glycerol, 1 mM-DTT and 1 mM-EDTA. During the elutions with 50 and 90 mM-potassium phosphate (500 ml), contaminating proteins were removed; pure ha *P*-450₁ was eluted with 0.25 M-potassium phosphate. The ha *P*-450₁ was dialysed overnight against 10 mM-potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 1 mM-DTT and 1 mM-EDTA and loaded on to a small hydroxyapatite column (1.5 cm × 2 cm). The column was washed with dialysis buffer until the 280 nm-absorbing material was removed.

The Emulgen 911-free ha *P*-450₁ was eluted with 0.25 M-potassium phosphate buffer, pH 7.7, containing 1 mM-DTT, 1 mM-EDTA, 20% (v/v) glycerol and 0.5% (w/v) sodium cholate, and extensively dialysed against 100 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol. The purified enzyme was stored at –80 °C in small aliquots.

The specific content of the ha *P*-450₁ preparation was 9.2 nmol/mg of protein. This low *P*-450 content could be due to the presence of apocytochrome ha *P*-450₁. Other preparations gave similar results.

Purification of other hepatic microsomal enzymes

Rat liver NADPH-cytochrome *P*-450 reductase (EC 1.6.2.4) was purified from phenobarbital (PB)-treated rats as described

by Yasukochi & Masters [44]. The preparation had a specific activity of 32 units/mg of protein (1 unit = 1 μ mol of cytochrome *c* reduced/min per mg protein). The enzyme activity was assayed according to the procedure of Masters *et al.* [45].

Hepatic cytochrome *b*₅ was purified from PB-treated rats by the method of Spatz & Strittmatter [46]. The purified cytochrome *b*₅ had a specific content of 32 nmol/mg of protein. Cytochrome *b*₅ and *P*-450 reductase were electrophoretically pure (results not shown).

Sequence analysis

Determination of *N*-terminal amino acid sequence was achieved by submitting the samples to automatic Edman degradation on a protein sequencer Model 470A (Applied Biosystems, Foster City, CA, U.S.A.) [47]. The separation and identification of the phenylthiohydantoin-amino acids was performed by an on-line PTH analyser Model 120A equipped with a control/data module 900A (Applied Biosystems).

Amino acid composition analysis

Amino acid composition was determined after acid hydrolysis in 6 M-HCl at 114 °C for 24 h in the Pico-Tag Work Station (Millipore Waters, Bedford, MA, U.S.A.) [48]. Derivatization of samples with phenyl isothiocyanate was performed by a pre-column procedure. Separation of phenylthiocarbamyl derivatives was carried out by reverse-phase h.p.l.c. using two model 510 pumps equipped with a Pico-Tag column, an absorbance detector model 441, an automated gradient controller and a Wisp 710B automatic sampler (all from Millipore-Waters).

Other methods

The protein concentration of the purification fractions was determined by the method of Lowry *et al.* [49], taking appropriate buffer controls and using BSA as standard. Cytochrome *P*-450 and haem content were determined as described by Omura & Sato [50]. SDS/PAGE was performed according to Laemmli [43] in separating gels (7.5% acrylamide) 0.75 cm thick and 10 cm long.

The immunoblot analysis of microsomal *P*-450 was performed according to previously published procedures [51]. Microsomes were subjected to SDS/PAGE followed by electrophoretic transfer to a nitrocellulose sheet at 200 V for 1 h. After rinsing with phosphate-buffered saline (PBS), the nitrocellulose was blocked with 3% (w/v) BSA in PBS and then incubated overnight with the antibodies against rat *P*-450, at room temperature. The nitrocellulose was then treated with a peroxidase-conjugated goat anti-(rabbit IgG) for 1 h. For immunostaining, the sheet was developed with 4-chloro-1-naphthol and H₂O₂.

The catalytic activity of ha *P*-450, was assayed under saturating conditions of NADPH and NADPH-*P*-450 reductase and at an optimal dilauroylphosphatidylcholine concentration in which metabolism was proportional to haemoprotein concentration and time of incubation. The system was generally reconstituted as follows: 0.1 μ M-ha *P*-450, 0.3 μ M-NADPH-cytochrome *P*-450 reductase and 30 μ g of dilauroylphosphatidylcholine/ml added as freshly sonicated micelles. The individual components of the reconstituted system were mixed and incubated for 30 min at room temperature before adding NADPH (1 mM) or an NADPH-generating system and substrate in a final volume of 1 ml of 100 mM-potassium phosphate buffer, pH 7.4. Formaldehyde derived from DMN, benzphetamine, ethylmorphine and aminopyrine was quantified according to Tu & Yang [52]. Aniline *p*-hydroxylase (AnH) activity was determined by measuring the amount of *p*-aminophenol formed, as described by Ko *et al.* [53]. 7-Ethoxycoumarin *O*-de-ethylase (ECOD) activity was assayed by the fluorimetric determination of 7-hydroxycoumarin,

according to the method of Aitio [54]. 7-Ethoxyresorufin *O*-de-ethylase activity was measured by the formation of the resorufin in a Perkin-Elmer spectrofluorimeter [55]. The extent of DEN de-ethylation was determined by h.p.l.c. by measuring acetaldehyde formation as its hydrazone derivative according to Farrelly [56], as previously reported in detail [57].

Acetone hydroxylase (AcH) activity was assayed by measuring the formation of acetol as its 2,4-dinitrophenylhydrazone derivative according to the h.p.l.c. method of Casazza & Fu [58], with slight modifications as previously reported in detail [21]. The assay for the oxidation of thiobenzamide to thiobenzamide *S*-oxide was performed as described by Cashman & Hanzlik [59].

RESULTS

Purification of ha *P*-450₁

As we previously reported [38], treatment of hamsters with acetone resulted in the induction of a microsomal protein with an apparent *M*_r of 55 000 in the microsomes of control animals. This protein has now been purified by chromatography on n-octylamine Sepharose 4B, DEAE-cellulose (DE 52) and hydroxyapatite as detailed in the Experimental section. The SDS/PAGE pattern, the specific content of *P*-450 and the binding to DMSO were used as criteria for the purification. A summary of a representative purification experiment is shown in Table 1.

As seen in Fig. 1, ha *P*-450, was separated from the majority of the microsomal proteins by hydrophobic chromatography on n-octylamine Sepharose 4B, a method previously developed for *P*-450, from pyrazole- or isoniazid-treated rats [8,10]. Based on SDS/PAGE, only four or five major contaminants co-elute with ha *P*-450. The majority of these contaminating proteins were removed from the *P*-450 preparation by chromatography on a DEAE-cellulose column, where ha *P*-450, along with few other proteins, did not absorb to anion-exchange cellulose at pH 7.25. At this stage the yield was about 4.4% (Table 1). Other contaminants were removed by chromatography on a DE 52 column at pH 7.7. At the higher pH, ha *P*-450, bound to the column, whereas other proteins were eluted in the flow-through.

The remaining contaminants were removed by stepwise elution from a hydroxyapatite column with 50 and 90 mM-potassium phosphate buffer. The ha *P*-450, which was eluted with 250 mM-potassium phosphate, was apparently electrophoretically pure, as judged by SDS/PAGE. It co-migrated with the protein of *M*_r 55 000 from acetone-induced microsomes. The final preparation after detergent removal showed a specific content of 9.2 nmol of *P*-450/mg of protein; the overall yield of the purification procedure was 1.1% (see Table 1).

Spectral properties

Fig. 2 illustrates the absolute spectrum of ha *P*-450, in the oxidized, dithionite-reduced and the CO-complexed states. Surprisingly, the absolute spectrum of ha *P*-450, did not show the characteristics of a high-spin protein, as observed for CYP2E1 in rat, rabbit and human [8,19,28]. In both the reduced and the oxidized state the absolute spectrum of ha *P*-450, displayed a maximum at 417 nm, similar to other low-spin microsomal *P*-450 isoenzymes [60]. In some preparations a shoulder at 385 nm was visible. The protein showed a mixed-type spectrum only in the high-ionic-strength buffers (2 M-KCl) (results not shown).

The CO-reduced difference spectral maximum of the enzyme was at 451–452 nm, as found for the CYP2E1(s) [8,19,28]. We previously reported [39] that DMSO, which is known to bind specifically to CYP2E1 in the rat [10] and rabbit [15], is bound by hamster hepatic *P*-450s, giving rise to a reverse type I difference spectrum both in control and in acetone-induced microsomes.

Table 1. Purification of ha *P*-450₁ from liver microsomes of acetone-treated hamsters

Abbreviation = HA, hydroxyapatite.

Fraction	Volume (ml)	Protein (mg/ml)	Total <i>P</i> -450 (nmol)	Specific <i>P</i> -450 content (nmol/mg of protein)	DMSO binding ΔA /nmol of <i>P</i> -450	Purification (fold)	Yield (%)
Solubilized microsomes	640	2.64	2278	1.35	0.023	1	100
Octylamine-Sepharose 4B fraction II eluate	900	0.13	346	2.95	0.042	2.2	15.2
DE 52 pH 7.25 flow-through eluate	160	0.18	100	3.47	0.048	2.6	4.4
DE 52 pH 7.7 fraction B eluate	80	0.09	35	4.86	0.053	3.6	1.5
HA 0.25 M-potassium phosphate eluate	10	0.36	25	9.2	0.066	6.8	1.1

Fig. 1. Gel electrophoresis of microsome proteins and ha *P*-450₁ at various stages of purification

Lane 1, control hamster liver microsomes; lane 2, acetone-induced microsomes; lane 3, fraction II eluted from n-octylamine-Sepharose 4B column; lane 4, flow-through fraction from the DE 52 column at pH 7.25; lane 5, 0.25 M-potassium phosphate eluate from hydroxyapatite column; lane 6, fraction B from the DE 52 column at pH 7.7.

The binding was biphasic, indicating a different affinity of the *P*-450 isoenzymes towards DMSO. Acetone pretreatment of animals enhanced only the amplitude of the binding spectrum related to the high-affinity component of DMSO binding, having a K_s of about 30 mM [38]. The measurement of the amplitude of this component of the DMSO-binding spectrum, as determined by Lineweaver-Burk plots with different DMSO concentrations (7–100 mM), in the samples of the various steps of the purification is another valid way to monitor the content of ha *P*-450₁ (Table 1). By contrast, the low-affinity component of the DMSO-binding spectrum of microsomes was lost after the first step of purification with the octylamine 4B chromatography.

Catalytic activities of ha *P*-450₁ in a reconstituted system

The catalytic activity of ha *P*-450₁ towards several substrates in a reconstituted mono-oxygenase system with and without cytochrome b_5 was compared with that of acetone-pretreated hamster liver microsomes (Table 2). This haemoprotein had low activity for the *O*-de-ethylation of 7-ethoxyresorufin, which is an activity typically markedly induced by 3-methylcholanthrene-type inducers not only in rats [40] but also in hamsters [61].

Low but measurable metabolism of 7-ethoxycoumarin was catalysed by ha *P*-450₁. The enzyme also effectively catalysed the

Fig. 2. Absolute spectra of ha *P*-450₁

The concentration of the cytochrome, based on the haem content, was 3.1 μ M in 100 mM-potassium phosphate buffer, pH 7.25, 20% glycerol, 0.1 mM-EDTA and 0.2% sodium cholate. The spectra were recorded at room temperature. Spectra are for oxidized *P*-450 (—), dithionite-reduced *P*-450 (---), and CO-ferrocyanide *P*-450 (···).

demethylation of aminopyrine, ethylmorphine and benzphetamine, but their catalytic-centre activities were not very different from those of acetone-induced microsomes (at most 2-fold higher).

On the other hand, ha *P*-450₁ was active in the hydroxylation of acetone and aniline, as well as in the *S*-oxidation of thio-benzamide; the rates were about 5–6-fold higher in the re-constituted system than in acetone-induced microsomes.

We recently demonstrated that acetone pretreatment in the hamster did not induce the high-affinity form of DENd, in contrast with rats and mice [21–23]. Microsomes from acetone-induced hamsters showed only an induction of the $V_{max,2}$ corresponding to the low-affinity form of DENd [39]. Therefore we investigated the ability of ha *P*-450₁ to de-ethylate DEN at two substrate concentrations: 0.5 mM, saturating the low- K_m DENd activity, and 5 mM, saturating both the high- and low- K_m DENd activities. As can be seen in Table 2, ha *P*-450₁ showed only low DENd activity when DEN was present at low concentrations; in the reconstituted system the specific activity was only 2-fold higher than in Ac-microsomes (microsomes from acetone-treated hamster liver). In contrast, ha *P*-450₁ was highly active

Table 2. Catalytic activity of ha P-450_j and liver microsomes from acetone-treated hamsters

The catalytic activities were measured as described in the Experimental section. The concentration of each substrate is indicated in parentheses and the concentration of cytochrome *b*₅, when used, was 0.1 μM. The data are averages of two or three experiments.

Substrate		Product determined	Activity (nmol/min per nmol of P-450)		
			Ac-microsomes	Ha P-450 _j	
				Without <i>b</i> ₅	With <i>b</i> ₅
Acetone	(10 mM)	Acetol	5.7	25.0	30.6
Aniline	(2.5 mM)	<i>p</i> -Aminophenol	2.8	14.5	10.7
7-Ethoxycoumarin	(100 μM)	7-Hydroxycoumarin	5.1	0.3	0.3
7-Ethoxyresorufin	(2 μM)	Resorufin	0.1	0.1	0.1
Aminopyrine	(8 mM)	Formaldehyde	7.6	13.0	21.2
DMN	(0.5 mM)	Formaldehyde	1.9	2.3	12.5
DMN	(4 mM)	Formaldehyde	2.6	5.6	15.7
DEN	(0.5 mM)	Acetaldehyde	1.6	3.2	3.9
DEN	(5 mM)	Acetaldehyde	6.9	61.0	68.9
Ethylmorphine	(1 mM)	Formaldehyde	2.8	3.9	5.3
Benzphetamine	(1 mM)	Formaldehyde	4.8	7.8	12.4
Thiobenzamide	(0.5 mM)	Thiobenzamide S-oxide	2.7	17.1	25.6

Fig. 3. Effect of cytochrome *b*₅ on DMNd activity

The incubation mixture contained 0.1 nmol of ha P-450_j, 0.3 nmol of P-450 reductase, 22 μg of dilauroylphosphatidylcholine and various amounts of cytochrome *b*₅ in a final volume of 0.75 ml. The incubation was performed at 37 °C for 10 min with 4 mM-DMN.

towards 5 mM-DEN, showing a specific activity of 61 nmol of acetaldehyde formed/min per nmol of P-450 (9-fold higher than in Ac-microsomes). The presence of cytochrome *b*₅ in the incubation mixture, except in the case of DMNd, did not markedly affect the oxidation rates of the substrates assayed. Similar slight effects of cytochrome *b*₅ on the same substrates have also been described for P-450_j [13].

Effects of cytochrome *b*₅ on the kinetic constants of DMNd

It has been reported that *b*₅ has a strong effect on the low-*K*_m activity of DMNd catalysed by P-450_j of the rat [13] and human [28]; thus we studied the effect of *b*₅ on the kinetics of DMNd catalysed by ha P-450_j. Upon addition of different amounts of *b*₅ to the reconstituted system the DMNd activity increased, reaching a plateau when the molar ratio of *b*₅/P-450 was 3:1 (Fig. 3). In the presence of this optimal molar ratio of *b*₅/P-450 in the reconstituted system, the apparent *K*_m value for DMNd decreased from 1.1 to 0.23 mM and the apparent *V*_{max} increased

Fig. 4. Lineweaver–Burk plots for DMNd activity in a reconstituted system

The incubation mixture (0.75 ml) contained 0.1 nmol of ha P-450_j, 0.3 nmol of P-450 reductase and 22 μg of phospholipid, without (●) or with (Δ) cytochrome *b*₅ (0.3 nmol). The incubations were performed at 37 °C for 10 min with various DMN concentrations.

from 6.2 to 19.4 nmol/min per nmol of P-450, as determined by Lineweaver–Burk plots (Fig. 4).

Influence of effectors on the *p*-hydroxylation of aniline

As ha P-450_j seemed to be a good catalyst of aniline hydroxylation, further characterization of this activity was carried out. As reported in Table 2, the AnH activity in a complete reconstituted system was 14.5 nmol/min per nmol of P-450. When dilauroylphosphatidylcholine was omitted from the incubation mixture, the AnH activity was halved. Without P-450 or NADPH–cytochrome P-450 reductase, no aniline hydroxylation was observed.

The effects of several inhibitors on AnH activity in microsomes and in the reconstituted system were studied (Table 3).

Table 3. Inhibition of reconstituted and microsomal AnH

The incubation mixture contained 0.1 nmol of *P*-450 in the reconstituted system as described in Fig 4, or control or Ac-microsomes (0.1 mg of protein). Inhibitors were added as aqueous or acetonitrile solutions. The results are expressed as percentage inhibition of control AnH activities, which were (nmol/min per nmol of *P*-450): control microsomes, 2.8; Ac-microsomes, 6.4; ha *P*-450_j, 14.5.

Inhibitor	Concn. (mM)	AnH activity (% inhibition)		
		Control microsomes	Ac- microsomes	ha <i>P</i> -450 _j
Thiobenzamide	1	54	60	73
	0.1	29	26	47
Diallylsulphide	1	34	32	53
	0.1	15	23	20
Disulfiram	1	34	36	42
	0.1	28	35	33
Diethyldithiocarbamate	1	44	56	53
	0.1	14	8	12

Metyrapone and imidazole at 1 mM did not inhibit this activity in either system (results not shown). Thiobenzamide and diethyldithiocarbamate were the most powerful inhibitors of AnH activity in both microsomes and ha *P*-450_j, although disulfiram and diallyl sulphide were also effective. In all cases the extent of inhibition of AnH activity in control or Ac-microsomes was similar to that in purified ha *P*-450_j, suggesting that this *P*-450 isoenzyme is selectively inhibited by these compounds.

N-Terminal amino acid sequence and amino acid composition of ha *P*-450_j

The *N*-terminal amino acid sequence for the first 34 residues of ha *P*-450_j, determined by automated Edman degradation, is shown in Fig. 5 and compared with that of rat *P*-450_j [62]. No other sequence was observed, indicating the purity of the haemoprotein preparation. Ha *P*-450_j began with an Ala residue and showed a sequence as hydrophobic as that of the rat (20 out of 34 residues), and 28 out of 34 amino acids were identical compared with the rat sequence (82% similarity). When the *N*-terminal sequence of ha *P*-450_j was compared with those of rabbit *P*-4503a [19] and human *P*-450_j [62], a lower degree of similarity was observed: 15 out of 25 and 23 out of 34 amino acids respectively.

The amino acid composition of ha *P*-450_j was determined (results not shown) and found to be similar to those of other *P*-450 isoenzymes, which contain 35–40% hydrophobic residues (Pro, Ala, Val, Met, Leu and Phe).

Fig. 6. Western blot analysis of ha *P*-450_j in liver microsomes from control and acetone-treated hamsters

Microsomes (5 µg of protein) were subjected to SDS/PAGE followed by electrophoretic transfer to nitrocellulose. The transfer membrane was then immunochemically stained with anti-(rat *P*-450_j) IgG. Lane 1, microsomes from β-NF-treated hamster; lane 2, ha *P*-450_j (0.5 pmol); lane 3, control microsomes; lane 4, Ac-microsomes. Lane 5 contains microsomes from acetone-treated rats.

Western blot analysis

Fig. 6 shows an immunoblot of microsomes prepared from untreated and treated hamsters and of purified ha *P*-450_j reacted with anti-(rat *P*-450_j) antibodies. In addition, in order to evaluate the specificity of the antibodies, microsomes from acetone-treated rats were also analysed. As we reported previously [38], acetone treatment of hamsters resulted in an increase of a protein with an *M_r* of approx. 55000. This protein cross-reacted with anti-(rat *P*-450_j) antibodies on immunoblotting, and corresponded to purified ha *P*-450_j. This band was present in control microsomes and increased after acetone induction. In β-NF-treated microsomes, ha *P*-450_j appeared to be depressed, as the intensity of this band was decreased with respect to control microsomes. In the rat, in agreement with previous data [10,37], a band of *M_r* 53000, representing *P*-450_j, was apparent in acetone-induced microsomes.

DISCUSSION

In the present study, by using methods similar to those employed in the purification of rat *P*-450_j [8,10], we have purified to homogeneity a *P*-450 isoenzyme, termed ha *P*-450_j, from the liver microsomes of acetone-treated hamsters and characterized some of its biochemical properties. This *P*-450, on the basis of its *N*-terminal amino acid sequence and biochemical features, is distinct from the few hamster *P*-450(s) so far purified. The *N*-terminal sequence of ha *P*-450_j shows little similarity to those of *P*-450 H and L forms [63] and of the unique *P*-450 MC-1 form, belonging to the CYP2A subfamily [64]. Moreover, the *P*450 protein obtained from PB-treated hamsters having an *M_r* of about 50000 and showing considerable ECOD activity [65], does

Fig. 5. Comparison of the *N*-terminal sequence of hamster *P*-450_j with that of rat *P*-450_j

not correspond to ha *P*-450₁, which has an M_r of 55000 and is a poor catalyst of ethoxycoumarin de-ethylation. Thus ha *P*-450₁ appears to be a novel hamster liver haemoprotein of M_r 55000 with a high similarity to CYP2E1 of other species [8,19,28].

Unlike these CYP 2E1, ha *P*-450₁ was purified predominantly in the low-spin state, as indicated by its absorbance maximum at 417 nm. Such a spin difference could be due to a change in hydrophobicity and the size of an amino acid close to the sixth ligand of ha *P*-450₁, as reported for mutated *P*-450_{coh} [66]. The CO-reduced spectrum of ha *P*-450₁, however, displayed an absorbance maximum at 451 nm, as found for the CYP2E1 of other species. Comparison of the *N*-terminal sequence of ha *P*-450₁ with the sequence reported for rat liver *P*-450₁ revealed that 28 of the first 36 amino acid residues are identical [62].

Ha *P*-450₁ was found to be catalytically very active towards aniline, acetone, thiobenzamide, DEN (5 mM) and DMN (0.5 mM), and the catalytic-centre activities were increased many-fold over those found with microsomes from acetone-treated hamsters.

Ha *P*-450₁ interacted with DMSO to produce a reverse type I binding spectrum, which showed the same K_s as the high-affinity component of the DMSO biphasic binding found in microsomes from acetone-treated hamsters [38].

Of remarkable significance was the finding that the presence of cytochrome b_5 in the reconstituted system, for the oxidation of DMN but not for other substrates [13], had a large effect: the apparent K_m was decreased by a factor of 5 from 1.1 to 0.23 mM. This latter value is quite dissimilar from the K_m value (36 μ M) described for the highest affinity component of DMNd activity with liver microsomes from weanling untreated hamsters [38]. As shown in the rat [13] and human [28], the high-affinity component of DMN demethylation, catalysed by ha *P*-450₁, is markedly affected by cytochrome b_5 . The discrepancy in the K_m values between ha *P*-450₁ and microsomes, also observed between rat microsomes and *P*-450₁ [13,67], could be a consequence of the presence of some inhibitors (e.g. glycerol) in our reconstituted system.

With regard to DEN metabolism, it is worth noting that ha *P*-450₁ catalysed with a high turnover only the low- and not the high-affinity component of DENd activity, in agreement with the results obtained with control and acetone-induced microsomes from hamster liver [39]. In contrast, *P*-450₁ was considered to be mainly responsible for the high-affinity component of microsomal DENd activity in acetone-induced and control rats [21,23]. It is probable that this is also true in mice [22]. It can be concluded from these findings that the substrate selectivities and the catalytic effectiveness of ha *P*-450₁ do not strictly parallel those described for rat *P*-450₁ [8,10,13].

Antibodies raised against rat *P*-450₁ recognized in both control and (more strongly) acetone-induced microsomes, one protein of M_r 55000 whose mobility is, on SDS/PAGE, identical to that of purified ha *P*-450₁.

Since it is known that hamster liver responds to xenobiotic treatment in a manner that is both qualitatively and quantitatively different from other species [61], it appears of interest to determine whether ha *P*-450₁ is inducible by the same acetone-like agents that induce CYP2E1 in other rodents. The hamster, furthermore, owing to the higher constitutive level of CYP2E1 compared with the rat (in which *P*-450₁ accounts for about 5–6 % of total *P*-450 [68]), represents a suitable animal model in which to study the regulation of this *P*-450 isoenzyme and the suggested presence of an endogenous inducer or stabilizer [69].

In summary, this paper describes the purification of a *P*-450 isoenzyme, designated ha *P*-450₁, from the liver microsomes of acetone-treated hamsters. Ha *P*-450₁ appears to be closely related to rat liver *P*-450₁, on the basis of several criteria, including

chromatographic, spectral, catalytic, immunochemical and structural characteristics. Therefore ha *P*-450₁ could represent the hamster orthologue of CYP2E1 of other species, and is the first member of the hamster CYP2E subfamily to be described.

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