Biochemical Pharmacology, Vol. 38, No. 2, pp. 235–240, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INTERSPECIES HOMOLOGY OF LIVER MICROSOMAL CYTOCHROME P-450

A FORM OF DOG CYTOCHROME P-450 (P-450-D1) CROSSREACTIVE WITH ANTIBODIES TO RAT P-450-MALE

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(*Received* 25 *November* 1987; *accepted* 22 *June* 1988)

Abstract—P-450-male is a male specific form of cytochrome P-450 in rat liver microsomes. Cytochrome P-450 crossreactive with anti-P-450-male antibodies was purified to an electrophoretical homogeneity from liver microsomes of male beagle dogs. The specific content of the purified cytochrome P-450 (P-450-D1) was 16.9 nmol/mg protein. The apparent monomeric molecular weight of P-450-D1 was 48,000, which was smaller than P-450-male (51,000). P-450-D1 showed similarities in spectral properties, N-terminal amino acid sequence, and catalytic activities with some limited exceptions: P-450-D1 did not catalyze 2α-hydroxylation of testosterone and progesterone and catalyzed 21-hydroxylation of progesterone. Based on these results, we propose that P-450-D1 is a form of cytochrome P-450 in the same gene subfamily as P-450-male.

Cytochrome P-450 in liver microsomes metabolizes a variety of substrates including endogenous and exogenous compounds. The apparent broad substrate specificities of cytochrome P-450 have recently been accounted for in part by the fact that there are multiple forms of cytochrome P-450: each form has considerable substrate specificity [1–4].

Forms of cytochrome P-450 may be classified into two groups: constitutive forms and forms inducible by foreign compounds. With respect to the interspecies similarities of cytochrome P-450, it is of interest to note the remarkable sequence as well as functional homology among certain forms of cytochrome P-450 from different animal species treated with the same inducer. Despite these studies, the interspecies homology of a constitutive form of cytochrome P-450 has not been examined to our knowledge.

P-450-male† is one of the constitutive forms of cytochrome P-450, and functions as 16α- and 2α-hydroxylases of testosterone [3, 5–10]. This cytochrome exists specifically in liver microsomes of adult male but not female rats. In adult female rats, P-450-female, which does not hydroxylate testosterone at 16α- and 2α-positions at detectable levels, is present [5, 11]. Although the catalytic activities of P-450-male and P-450-female differ remarkably, these cytochromes are found to show relatedness in immunochemical crossreactivity and N-terminal amino acid sequences, suggesting that these cytochromes are derived from the same ancester gene [5, 11]. In another study, we found that the crude preparation of anti-P-450-male antibodies crossreacted with a protein(s) in liver microsomes from other animal species including beagle dogs.‡ The antibodies inhibited testosterone hydroxylase in all animal species, although the hydroxylases inhibited by the antibodies varied depending on the animal species.‡

The purpose of the present study was, thus, to purify a form of cytochrome P-450 crossreactive with anti-P-450-male antibodies to find out whether a form of cytochrome P-450 corresponding to P-450-male exists in liver microsomes of male beagle dogs. We report herein that the purified preparation of beagle dog cytochrome P-450 (P-450-D1) may be in the same gene subfamily as P-450s f [8], g [8], h (= male), i (= female) [8, 11], and PB1 [9].

MATERIALS AND METHODS

Materials. Emulgen 911, a nonionic detergent, was a gift from Kao-Atlas, Tokyo, Japan. An ω-amino-*n*-octyl derivative of Sepharose 4B was prepared from 1,8-diaminooctane (Aldrich) and Sepharose 4B [12]. Hydroxylapatite (Bio Gel HT) was purchased from Bio-Rad. Preparative and analytical DEAE-5PW columns were obtained from Toyo Soda, Tokyo, Japan. 7-Ethoxycoumarin was purchased from Aldrich. NADPH–cytochrome P-450 reductase was purified from liver microsomes of phenobarbital-treated rats as previously described [13]. P-450-male

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† Abbreviations: P-450-male, a form of male-specific cytochrome P-450 [5] in rat liver microsomes corresponding to preparations designated as $P\text{-}450_{UT\text{-}A}$ [3], P-450(M1) [6], RLM5 [7], P-450h [8], P-450 2c [9], and P-450 A [10] in other laboratories; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; and DEAE, diethylaminoethyl.

‡ T. Miura, M. Shimada, M. Komori, T. Kodama and T. Kamataki, manuscript submitted for publication.

(UT-2) was purified from liver microsomes of male rats by the method of Funae and Imaoka [14] with minor modifications. The specific content of P-450-male was 16.4 nmol/mg protein. Other chemicals used were of the highest reagent grade available.

Analytical and assay methods. Protein was determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard. The content of cytochrome P-450 was estimated from the carbon monoxide difference spectrum described by Omura and Sato [16]. Polyacrylamide slab gel electrophoresis was performed by the method of Laemmli [17] using 7.5% polyacrylamide. The gel was fixed by 50% methanol and then stained [18]. Western blot analysis using anti-P-450-male antibodies was carried out by the method of Towbin *et al.* [19] as modified by Guengerich *et al.* [20]. The N-terminal sequence of the purified P-450-D1 was determined with Applied Biosystem gas phase protein sequencer. NADPH-cytochrome P-450 reductase was assayed by measuring the reduction of cytochrome *c* from NADPH. The activity of the reductase was defined as a unit which represents 1 μmol of cytochrome *c* reduced/min [13]. A typical incubation mixture for the assay of steroid and drug-metabolizing enzymes consisted of an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and 5 mM magnesium chloride), 100 mM sodium potassium phosphate (pH 7.4), and a substrate (0.1 mM testosterone, 0.1 mM progesterone, 5 mM aniline, 5 mM benzphetamine, 0.5 mM 7-ethoxycoumarin, or 5 mM aminopyrine) in a final volume of 1.0 ml. The incubations were started by the addition of the NADPH-generating system. Testosterone and progesterone hydroxylase activities were estimated by analyzing the metabolites using HPLC.* Activities of aniline *p*-hydroxylase, benzphetamine and aminopyrine *N*-demethylase, and 7-ethoxycoumarin *O*-deethylase were assayed by determination of *p*-aminophenol [21], formaldehyde [22], and 7-hydroxycoumarin [23] respectively.

Purification of P-450-D1 from liver microsomes of male beagle dogs. Liver microsomes from male beagle dogs were solubilized with 100 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) sodium cholate, 1 mM DTT, 0.1 mM EDTA and 20% glycerol as described previously [24]. The solubilized supernatant fraction was applied to an ω-amino-*n*-octyl Sepharose 4B column (26 × 700 mm) equilibrated with the solubilizing buffer. For each gram of microsomal protein, 200–300 ml of the resin was used. The column was washed with 1–1.5 times the column volume of 10 mM potassium phosphate buffer(pH 7.4) containing 0.5% sodium cholate, 1 mM DTT, 0.1 mM EDTA and 20% glycerol. When the first peak of cytochrome P-450 was eluted with 2–3 times the column volume of the same buffer containing 0.2% Emulgen 911 (Buffer A), a linear gradient elution was started from Buffer A to 200 mM potassium phosphate buffer (pH 7.4) containing 0.5% sodium cholate, 0.5% Emulgen 911, 1 mM DTT, 0.1 mM EDTA and 20% glycerol (Buffer B). The fractions, which gave positive signals in Western blot analysis with anti-P-450-male antibodies, were pooled. The pooled fraction was concentrated to give 45–65 μM cytochrome P-450 using an ultrafiltration membrane (UK-50, Toyo Roshi International Inc., Japan), and then diluted 10-fold with 20 mM Tris–acetate (pH 7.5) containing 20% glycerol. This sample was applied to HPLC equipped with a preparative DEAE-5PW column (21.5 × 150 mm) equilibrated with 20 mM Tris–acetate (pH 7.5) containing 0.4% Emulgen 911 and 20% glycerol (Buffer C). After the pass-through fraction was eluted, a linear gradient elution of sodium acetate from 0 to 1 M (Buffer D) was started according to the method of Funae and Imaoka [14]. The flow rate was set at 2.0 ml/min, and the elution of the hemoprotein was monitored at 405 nm. The pooled eluates, which showed crossreactivity with anti-P-450-male antibodies, were concentrated to give about 20 μM cytochrome P-450, diluted 5-fold with 20 mM Tris–acetate (pH 7.5) containing 20% glycerol, and then applied to HPLC equipped with an analytical DEAE-5PW column (7.5 × 74 mm) [14]. To remove Emulgen 911, the eluates from an analytical DEAE-5PW column were applied to a hydroxylapatite column (5 × 20 mm) equilibrated with 10 mM sodium phosphate (pH 7.4) containing 20% glycerol. The column was washed with the same buffer until the absorption at 280 nm due to Emulgen 911 was not detectable. Cytochrome P-450 was eluted with 300 mM sodium phosphate (pH 7.4) containing 20% glycerol.

RESULTS AND DISCUSSION

Purification of P-450-D1. The elution profile of cytochrome P-450 from an ω-amino-*n*-octyl Sepharose column is shown in Fig. 1. The fractions indicated as a shaded area in the figure gave positive signals in the Western blot analysis with anti-P-450-male antibodies. The pooled fraction contained

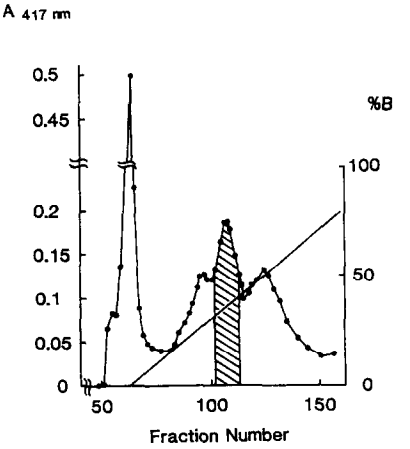

Fig. 1. Elution profile of cytochrome P-450 from an amino-octyl Sepharose column. When the first peak of cytochrome P-450 was eluted with buffer A, a linear gradient elution (buffer A to B) was started. For further details, see Materials and Methods. Each fraction contained 15 ml of the eluate.

‡ T. Miura, M. Shimada, M. Komori, T. Kodama and T. Kamataki, manuscript submitted for publication.

Table 1. Summary of a typical purification of P-450-D1 from liver microsomes of untreated male dog

Purification step	Total protein (mg)	Cytochrome P-450		
		Total (nmol)	S.C.* (nmol/mg protein)	Recovery (%)
Microsomes	1480	1120	0.76	100
Solubilized supernatant	1120	1030	0.92	92
Aminooctyl Sepharose eluate	63	130	2.1	11
1st DEAE-5PW eluate	0.91	11.8	13.0	1.1
2nd DEAE-5PW eluate	0.42	7.1	16.9	0.63

The purification was carried out as described in Materials and Methods.
* Specific content of cytochrome P-450.

about 11% of the microsomal cytochrome P-450, and the specific content of cytochrome P-450 was 2.1 nmol/mg protein (Table 1). Panels (a) and (b) of Fig. 2 show the HPLC elution profiles of cytochrome P-450 as monitored by the absorption at 405 nm from the preparative and analytical DEAE-5PW columns respectively. Three cytochrome P-450 peaks were eluted by Buffer C and three more peaks eluted by a linear gradient of sodium acetate from the preparative DEAE-5PW column (Fig. 2a). The last peak (shaded peak) eluted by Buffer C gave strong positive crossreactivity with anti-P-450-male antibodies. The recovery of cytochrome P-450 cross-reactive with the antibodies (eluted in a shaded peak in the figure) was rather low (1.1%), whereas the specific content was increased up to 13.0 nmol/mg protein (Table 1). The pooled fractions still contained two major protein bands on SDS–PAGE. After the pass-through peak was eluted from the analytical DEAE-5PW column, cytochrome P-450 was eluted sharply in a linear gradient of sodium acetate at about 20 mM (Fig. 2b), which showed a single band on SDS–PAGE and crossreacted with anti-P-450-male antibodies. The final preparation of cytochrome P-450 was designated as P-450-D1.

Table 1 summarizes typical results of purification of P-450-D1 from liver microsomes of untreated male dogs. The recovery in the final P-450-D1 preparation was approximately 0.6% of the total cytochrome P-450 in the starting microsomes. The specific content of P-450-D1 was about 17 nmol/mg protein.

Purity and molecular properties of P-450-D1. The final preparation of P-450-D1 was essentially homogeneous (Fig. 3, lane 4) as judged by SDS–PAGE. The result of Western blot analysis at each purification step is shown in Fig. 4. Throughout the purification procedure, the protein band which crossreacted with anti-P-450-male antibodies existed at the same position as seen with microsomes. Figure 4 also shows that the apparent molecular weight of P-450-D1 is smaller than that of P-450-male. Comparing the electrophoretic mobility of P-450-D1 with those of standard proteins (Fig. 3, lane 5), the apparent molecular weight of P-450-D1 was estimated to be 48,000.

The spectral properties of P-450-D1 are shown in Fig. 5. In the oxidized state, it exhibited a Soret absorption peak at 414 nm together with a gentle shoulder near 390 nm. A small but significant peak was also detected near 645 nm. These indicate that

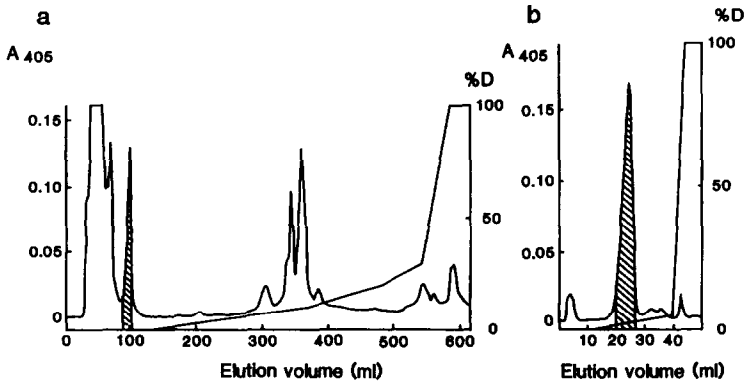

Fig. 2. HPLC elution profile of cytochrome P-450 using preparative (a) and analytical (b) DEAE-5PW columns. (a) Pooled fraction eluted from an aminooctyl Sepharose column was applied to HPLC equipped with a preparative DEAE-5PW column. Chromatography was done at a flow rate of 2 ml/min with a linear gradient of sodium acetate from 0 to 1 M. Hemoproteins were monitored at 405 nm. (b) The eluate fraction from a preparative DEAE-5PW column was rechromatographed by HPLC using an analytical DEAE-5PW column. Shaded peak fractions in panels (a) and (b) strongly crossreacted with anti-P-450-male antibodies.

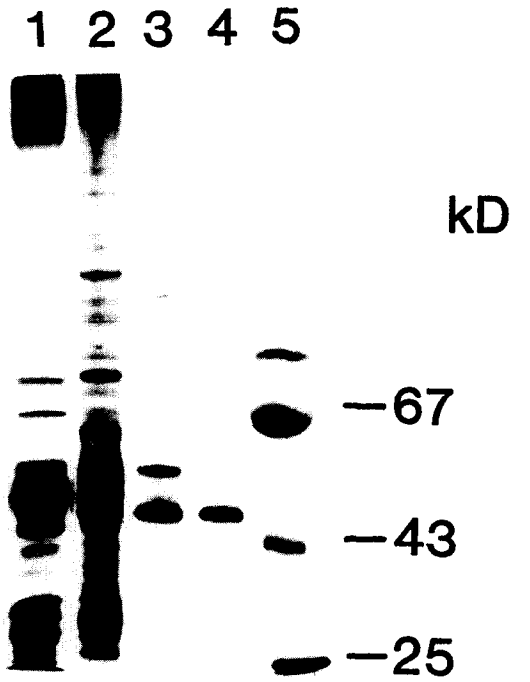

Fig. 3. SDS–PAGE of proteins according to purification steps. After being electrophoresed, the proteins were analyzed by silver staining. Molecular weight markers used were bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), and chymotrypsinogen A (M_r 25,000). Lane 1, liver microsomes (50 μg protein); lane 2, aminooctyl Sepharose eluates (25 μg); lane 3, preparative DEAE-5PW eluates (4 μg); lane 4, purified P-450-D1 (1.5 μg); and lane 5, standard proteins.

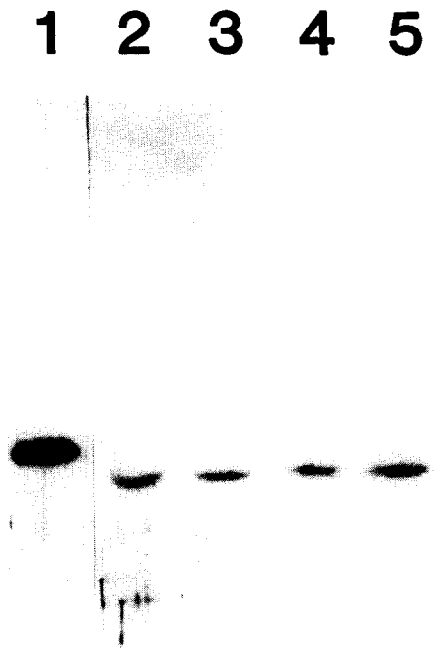

Fig. 4. Western blot analysis of proteins crossreactive with anti-P-450-male antibodies. Western blot-peroxidase staining analysis of proteins was carried out using anti-P-450-male antibodies. Lane 1, rat P-450-male (1.5 μg); and lane 2–5, the same samples (the same quantities of protein) as respective lanes 1–4 in Fig. 3.

the heme protein is mostly in a low spin state and a remaining small portion is in a high spin state. Such a feature is essentially consistent with those of P-450-male [5]. The carbon monoxide complex of reduced P-450-D1 showed absorption maxima at 451 nm, which is comparable to that of P-450-male (451.5 nm) [5].

It has been reported that there are at least five forms of cytochrome P-450 in the same gene subfamily as P-450-male in rat, i.e. forms of cytochrome P-450 designated as f, g, h (= male), i (= female) and PB1 [8, 9, 11]. These are all crossreactive immunochemically and all exhibit significant sequence similarities, even in the N-terminal region. The N-terminal amino acid sequence of P-450-D1 is shown in Fig. 6 together with those of various cytochromes in rat livers [8, 9, 11, 25–27]. About 56% identity was found between P-450-D1 and P-450-male [P-450(M-1)] or P-450f when calculated on the basis of the number of amino acids identified. The amino acid sequences in the amino terminal region have been recognized as not being conserved among various forms of cytochrome P-450; thus, a 56% identity probably suggests fairly high homology between P-450-D1 and P-450-male or P-450f. Supporting this view, only scant or no homology was seen when compared to the sequences of P-450b and P-450c. In the N-terminal sequence of P-450-D1, the proline-cluster region at 29–34 residues (L-P-P-G-P) was also found. The region has been known to be conserved in the phenobarbital-inducible forms of cytochrome P-450 in liver microsomes of rabbits [28].

Catalytic properties of P-450-D1. Testosterone and progesterone metabolizing enzyme activities of P-450-D1 and P-450-male are compared in Table 2. The activities in liver microsomes of male dogs and rats are also shown in the table. Liver microsomes from both animals were capable of forming 6β-

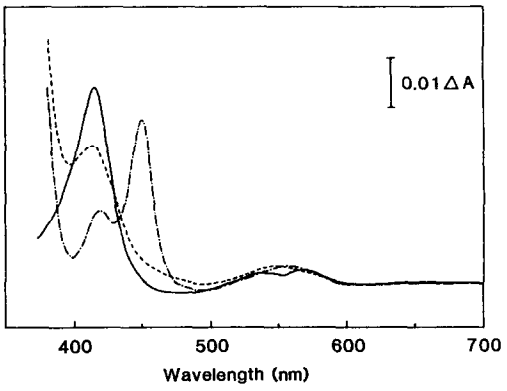

Fig. 5. Absolute absorption spectra of purified P-450-D1. The spectra of P-450-D1 in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.4% Emulgen 911 were measured: (——) oxidized form; (·····) dithionite-reduced form; and (-·-·-) carbon monoxide complex of reduced form.

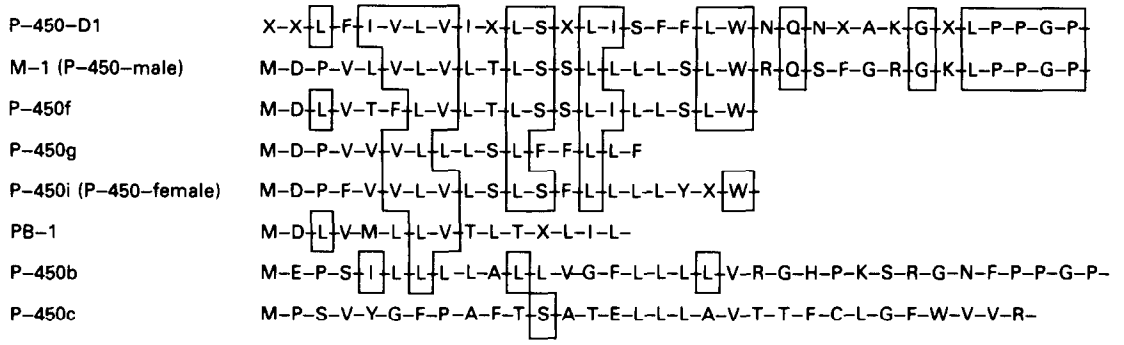

Fig. 6. Comparison of amino terminal sequences of P-450-D1 with various forms of cytochrome P-450 in rat liver. Amino acid sequences of three forms of cytochrome P-450 in rat liver were deduced from their nucleic acid sequences: P-450 (M-1) (P-450-male), male-specific form [25], P-450b, phenobarbital-inducible form [26], and P-450c, methylcholanthrene-inducible form [27]. Amino acid residues marked X of P-450-D1 were not identified, and those identical to P-450-D1 are boxed.

hydroxytestosterone, whereas the purified preparations of P-450-D1 and P-450-male were incapable of producing the metabolite, indicating that the hydroxylation at this position was catalyzed by a form(s) of cytochrome P-450 other than these cytochromes in these animals. Both of the purified cytochromes showed high 16α-hydroxylase activities of testosterone and progesterone in common. P-450-D1 did not catalyze the 2α-hydroxylation of testosterone, which was in agreement with the fact that the hydroxylation was not detectable in liver microsomes of beagle dogs. A similar tendency was seen in the 2α-hydroxylation of progesterone in beagle dogs. P-450-D1 was capable of hydroxylating progesterone at the 21-position at a relatively faster rate as compared to the rate in liver microsomes. These results suggest the major contribution of P-450-D1 in this reaction in liver microsomes of beagle dogs. The 21-hydroxylase activity of progesterone was not detectable when P-450-male and liver microsomes from male rats were examined. In addition to the 16α-hydroxylases of testosterone and progesterone, both P-450-D1 and P-450-male showed high activities to form androstenedione from testosterone (not shown) and fairly low activities of progesterone 6β-hydroxylase in common. This common nature of the purified preparations of cytochrome P-450 may support, at least in part, the view that P-450-D1 and P-450-male have similar properties.

It is known that the addition of cytochrome b_5 to the reconstituted system enhances the activity of cytochrome P-450. Thus, the effect of cytochrome b_5 on testosterone 16α-hydroxylase activity of P-450-D1 was examined in a preliminary experiment. The results showed that the hydroxylase activity was not enhanced but rather inhibited by cytochrome b_5 (by about 12%).

Table 3 shows the drug-metabolizing activities of liver microsomes and purified P-450-D1 and P-450-male. Liver microsomes from rats possessed the highest activity in aminopyrine *N*-demethylase, followed by benzphetamine *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and aniline hydroxylase. The same order of the activities was found in liver microsomes from beagle dogs, although the activities of the animals varied between the species. The highest activity was seen in benzphetamine *N*-demethylase, followed by aminopyrine *N*-demethylase, aniline

Table 2. Testosterone and progesterone hydroxylase activities of liver microsomes from beagle dogs and rats and purified preparations of P-450-male and P-450-D1 in reconstituted system

Substrate	Microsomes		Reconstituted system	
	Rat	Dog	P-450-male	P-450-D1
	(nmol/min/nmol cytochrome P-450)			
Testosterone				
2α-hydroxylation	2.00	<0.01	1.04	<0.01
6β-hydroxylation	2.04	1.87	<0.01	<0.01
16α-hydroxylation	1.94	0.55	2.41	4.20
Progesterone				
2α-hydroxylation	3.36	0.13	4.98	<0.01
6β-hydroxylation	0.45	2.63	0.24	0.36
16α-hydroxylation	3.36	0.44	3.74	2.76
21-hydroxylation	<0.01	0.13	<0.01	2.98

The reconstituted system contained P-450-D1 or P-450-male, NADPH–cytochrome P-450 reductase purified from rat liver microsomes, and dilauroyl phosphatidyl choline as described previously [5].

Table 3. Drug-metabolizing activities of liver microsomes from beagle dogs and rats and purified preparations of P-450-male and P-450-D1 in reconstituted system

Substrate	Microsomes		Reconstituted system	
	Rat	Dog	P-450-male	P-450-D1
	(nmol/min/nmol cytochrome P-450)			
Benzphetamine *N*-demethylation	13.8	7.17	46.3	21.7
Aminopyrine *N*-demethylation	16.1	8.18	10.7	11.5
7-Ethoxycoumarin *O*-deethylation	1.83	2.06	1.27	3.74
Aniline hydroxylation	1.13	0.47	2.50	7.54

The reconstituted system contained necessary components as in Table 2. The metabolites formed were analyzed as described elsewhere [21–23].

hydroxylase and 7-ethoxycoumarin *O*-deethylase in common in the reconstituted system containing either P-450-D1 or P-450-male. Compared to the activities in liver microsomes, relatively higher activities of P-450-D1 were seen in benzphetamine *N*-demethylation and aniline hydroxylation. Thus, with minor exceptions, it can be confirmed that P-450-D1 and P-450-male possess similar drug-metabolizing capacities.

We purified P-450-D1 from liver microsomes of male beagle dogs that was crossreactive with anti-P-450-male antibodies. The purified preparation showed similarities in the N-terminal amino acid sequence and catalytic activities besides the immunochemical crossreactivity. These results support the idea that P-450-D1 is a form of cytochrome P-450 belonging to the same gene subfamily as P-450-male. The possibility of whether P-450-D1 exists in liver microsomes of male beagle dogs in higher amounts than in female animals is of interest to examine in future studies. In addition, the comparison of total primary amino acid sequences between P-450-D1 and P-450-male is under investigation in this laboratory.

Acknowledgements—We are grateful to Drs. Funae and Imaoka for their suggestions concerning purification of cytochrome P-450 by HPLC. This work was supported in part by a Grant-in-Aid (No. 61480426) from the Ministry of Education, Science and Culture of Japan, and also by the Takeda Science Foundation, the Naitoh Foundation and the Kuribayashi Foundation for Education and Science.

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