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PURIFICATION AND CHARACTERIZATION OF A FORM OF CYTOCHROME P450 FROM BEAR LIVER MICROSOMES

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Abstract—A form of P450 [termed P450(b-1)] was purified from male bear liver microsomes. The specific content of the final P450(b-1) preparation was 11.26 nmol/mg protein, and recovery was 0.20% of the microsomal P450. The apparent molecular weight of P450(b-1) was 54,000. The absorption spectrum of P450(b-1) indicated that this protein was a low- and high-spin mixed type P450 in the oxidized form. The carbon monoxide complex of reduced P450(b-1) showed an absorption peak at 450.5 nm. The reconstituted system containing P450(b-1) catalyzed the metabolism of aminopyrine, benzo[*a*]pyrene, 7-ethoxycoumarin, imipramine and propranolol, of which P450(b-1) most strongly catalyzed aminopyrine N-demethylation and imipramine N-demethylation. The N-terminal amino acid sequence of P450(b-1) was highly homologous to that of P450-D1 from liver microsomes of male beagle dogs. P450(b-1) showed similarities in spectral properties, N-terminal amino acid sequence, and catalytic activities to rat P450 2C11. P450(b-1) was immunochemically cross-reactive with anti-P450 2C11 antibody and very weakly cross-reactive with anti-P450 2E1 antibody, but did not react with anti P450 1A1 or 2B1 antibodies. On the basis of these results, we suggest that P450(b-1) belongs to the P450 2C subfamily.

Key words: cytochrome P-450; purification; CYP2C; bear; *Canoidea*

Cytochrome P450 (P450) is the major catalytic component of the liver microsomal mixed-function oxidase system that catalyzes the biotransformation of endogenous substrates including steroids and fatty acids and exogenous substrates such as drugs, chemical carcinogens, and environmental pollutants [1]. During the past decade, more than 200 genes of P450 have been isolated and divided into 36 gene families, including 22 mammalian subfamilies based on the identity of the primary sequences [2]. In mammals, hepatic P450s belonging to at least 8 subfamilies are involved in the oxidative metabolism of xenobiotics.

Other than genetically based classification, forms of P450 may be classified into two groups (constitutive forms and inducible forms). With respect to the interspecies similarities of P450, it is of interest to note the remarkable sequence as well as functional homology among certain forms in different animal species treated with the same inducer [1,3]. With respect to the interspecies comparison of constitutive forms of P450, the CYP2‖ subfamily has been examined in detail among experimental animals and some domestic animals [4–8]. However, little is known about the hepatic drug-metabolizing enzymes of large wild animals. The number of brown bears (*Canoidea ursus*), the largest wild animal in Japan, has been decreased by developing forest land for agricultural or residential use. Brown bears belonging to the *Canoidea* superfamily are omnivorous and feed on other animals, fish (typically salmon), insects, and some fruits. Since they are at the top of the hierarchy of the food chain, they face a risk of accumulating environmental pollutants in their bodies. They have some notable features, e.g. hibernation and delayed implantation. Hepatic drug-metabolizing enzymes in brown bears also may be different from those of other animals. It is important to investigate and to determine their ability to metabolize xenobiotics: such knowledge will help to establish the susceptibility of large wild animals to xenobiotics; information regarding the structural and functional relationships of cytochrome P450 among different animal groups will help to elucidate some of the evolutionary aspects of this supergene family; and studies of P450 in these wild animals may help to establish pharmacological properties of therapeutic drugs that would aid in the development of specific species treatment modalities, especially in cases where disease may be endemic in the population, or in the treatment of animals in captivity.

In this study, we have purified a constitutive form of P450 [termed P450(b-1)] from bear liver

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‖ The nomenclature used in this report has been described by Nelson *et al.* [2].

microsomes and characterized its molecular, spectral, catalytic, and immunochemical properties.

MATERIALS AND METHODS

Materials. The livers of three adult male brown bears (7 years old) were supplied by the local fur industry (Hokkaido, Japan). A portion of each liver was dissected into 3-cm cubes and frozen immediately in liquid nitrogen. Frozen tissues were stored at −80° until used. The following chemicals and biochemicals were from the sources indicated in parentheses: octyl-Sepharose 4B (Pharmacia); Emulgen 911 (Kao-Atlus); and prestained protein molecular weight standards (GIBCO BRL). NADPH-cytochrome P450 reductase was purified from liver microsomes of phenobarbital-treated rats as previously described [9]. P450 UT-2(2C11) was purified from liver microsomes of male rats as previously described [5]. Other chemicals used were of the highest grade available.

Purification of P450 from bear liver microsomes. Bear liver microsomes were prepared according to a method reported for rat liver microsomes [10] with a slight modification. The diced liver was homogenized with 3 vol. of 1.15% KCl, initially minced with a Mixer (HM-7SA, Nippon Rikagaku Kikai, Japan) at 1000 rpm for 2 min, and then homogenized in a glass homogenizer with a teflon pestle. Liver homogenate was centrifuged at 9000 *g* for 20 min. The supernatant fraction was further centrifuged at 105,000 *g* for 90 min and the pellet was washed again with the same buffer. The microsomal preparation was diluted to 7.0 mg protein/mL with 0.1 M potassium phosphate buffer (pH 7.4) containing 30% glycerol, 1.0 mM EDTA, and 1.0 mM dithiothreitol. Sodium cholate solution (10%) was added to a final concentration of 3.0 mg/mg protein. After 30 min of stirring, 50% polyethylene glycol 6000 solution (50%) was added to a final concentration of 7% at 0°. The resulting suspension was stirred for 30 min more and then centrifuged at 35,000 *g* for 30 min. The supernatant was decanted, and then polyethylene glycol solution was added to the supernatant until a final concentration of 15% was reached. After 30 min of stirring, the suspension was centrifuged at 105,000 *g* for 60 min. The 7–15% polyethylene glycol precipitate was suspended in a final concentration of 5 mg protein/mL in 0.1 M potassium phosphate buffer (pH 7.2), containing 20% glycerol, 1 mM EDTA, and 0.5 mM dithiothreitol. This suspension was solubilized with the addition of sodium cholate to a final concentration of 0.7%. The resulting solution was put on an octyl-Sepharose 4B column (C26/40, 26 × 400 mm, Pharmacia) equilibrated with 0.1 M potassium phosphate buffer (pH 7.2), containing 20% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, and 0.5% sodium cholate. The column was washed with equilibration buffer. Cytochrome P450 was eluted with 0.1 M potassium phosphate buffer (pH 7.2), containing 20% glycerol, 1.0 mM EDTA, 0.5 mM dithiothreitol, 0.4% sodium cholate, and 0.1% Emulgen 911 (Kao, Tokyo, Japan). The eluate (300 mL) from the octyl-Sepharose 4B chromatography was concentrated to 30 mL, using an ultrafiltration membrane (UK-50, Advantec, Japan). The resulting solution was purified with an HPLC apparatus equipped with a preparative DEAE-5PW anion-exchange column (DEAE-5PW, 21.5 × 150 mm, Tosoh, Japan). Chromatography was done at a flow rate of 2 mL/min with a linear gradient of sodium acetate from 0 to 0.2 M over 180 min in 20 mM Tris-acetate buffer (pH 7.5), containing 20% glycerol and 0.4% Emulgen 911. Hemoprotein was monitored at 417 nm. The eluted cytochrome P450 was further purified by HPLC with a hydroxyapatite column (HAC-5CP, JASCO, Japan). The chromatography was carried out at a flow rate of 0.5 mL/min with a linear gradient from 0.01 to 0.35 M sodium phosphate buffer (pH 7.4), containing 20% glycerol and 0.2% sodium cholate over 70 min. All HPLC was done at room temperature.

Analytical methods. The contents of P450 and protein were determined by the methods of Omura and Sato [11], and Lowry *et al.* [12], respectively. Spectrophotometric measurements were carried out with an HITACHI U-3300 spectrophotometer. SDS–PAGE was carried out according to the method of Laemmli [13], using 10% polyacrylamide. Proteins separated by SDS–PAGE were blotted onto a nitrocellulose membrane and immunostained with diaminobenzidine as substrate [14]. Rat P450 2C11 [5], 1A1 [5], 2B1 [5], and 2E1 [15] antibodies were raised in rabbits as described previously. Determination of the N-terminal amino acid sequence of purified P450 was carried out as follows [16]. Purified P450 was subjected to SDS–PAGE (10% gel), and electroblotted on a polyvinylidine difluoride (Imobilon-P, Millipore) membrane, and the blotted portion of the membrane was cut out. The cut-out portion was applied to a liquid-phase protein sequencer (Applied Biosystems 477A).

Assay methods. NADPH-P450 reductase activities were measured using cytochrome *c* as an electron acceptor by the method of Van Gelder and Slater [17]. A typical incubation mixture for the assay of drug-metabolizing enzymes consisted of an NADPH-generating system (0.5 mM NADPH, 10 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 5 mM magnesium chloride), 100 mM potassium phosphate buffer (pH 7.4), and a substrate (0.1 mM 7-ethoxycoumarin, 1 mM aminopyrine, 1 mM imipramine, 1 mM propranolol) in a final volume of 1.0 mL. The incubations were started by the addition of NADPH and glucose-6-phosphate dehydrogenase. The reconstituted system contained 50 nM P450, NADPH-P450 reductase (0.4 U/mL), dilauroylphosphatidylcholine (10 μg/mL), 0.1 mM NADPH, 100 mM phosphate buffer (pH 7.2), and a substrate in a final volume of 0.5 mL. Aminopyrine *N*-demethylase activity was measured by the determination of formaldehyde [18]. Benzo[*a*]pyrene 3-hydroxylase activity was measured using the method of Nebert and Gelboin [19]. The fluorescence of the alkaline extract was assayed at an excitation wavelength of 396 nm and an emission wavelength of 522 nm using a JASCO FP-777 spectrofluorometer calibrated with a quinine sulfate and converted into amounts of 3-hydroxybenzo[*a*]pyrene using the factor given by Uemura and Chiesara [20]. 7-

Table 1. Purification of P450(b-1) from bear liver microsomes

Purification step	Protein (mg)	P450 Total (nmol)	P450 S.C.* (nmol/mg)	P450 Yield (%)
Microsomes	5173	1628	0.31	100.0
PEG fractionation	2763	1172	0.42	72.0
Octyl-Sepharose 4B	211	535	2.53	32.8
DEAE-5PW	3.58	17.21	4.80	1.06
Hydroxyapatite	0.29	3.30	11.26	0.20

Purification was carried out as described in Materials and Methods.
* S.C., specific content of cytochrome P450.

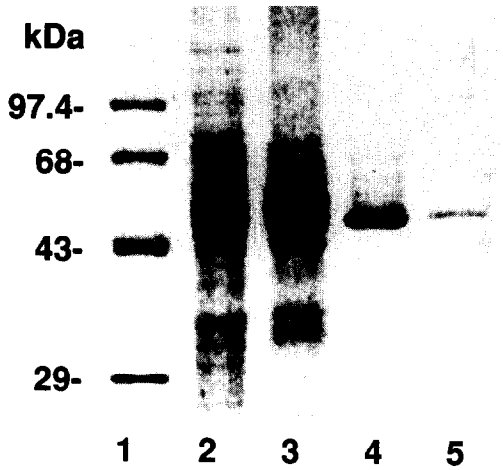

Fig. 1. SDS–PAGE of proteins according to purification steps. After being electrophoresed, the proteins were analyzed by silver staining. Lane 1, standard protein; lane 2, 10 μg of bear liver microsomes; lane 3, 5.0 μg of octyl-Sepharose eluate; lane 4, 2.5 μg of DEAE-5PW eluate; and lane 5, 1.0 μg of purified P450(b-1). Molecular standard markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

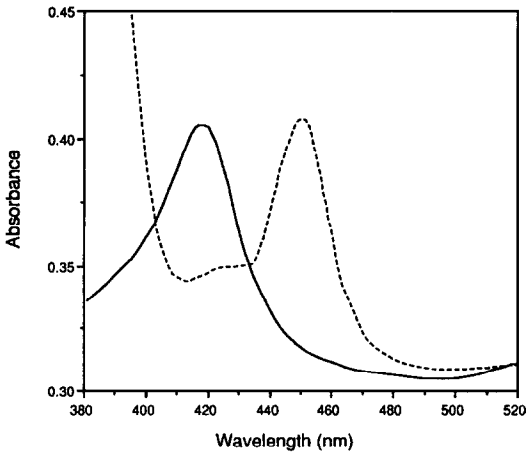

Fig. 2. Absolute absorption spectra of purified P450(b-1) measured in 20 mM sodium phosphate buffer (pH 7.2), 20% glycerol, 0.2% sodium cholate. The solid line indicates the absorption spectrum of the oxidized form, and the broken line, carbon monoxide complex of the dithionite-reduced form.

Ethoxycoumarin *O*-deethylase activity was assayed as described previously [21]. Imipramine 2-hydroxylase and *N*-demethylase, and propranolol 4-hydroxylase, 5-hydroxylase and 7-hydroxylase activities were measured by HPLC as described previously [22,23].

RESULTS AND DISCUSSION

Purification of a form of P450 from bear liver microsomes. Table 1 summarizes the purification steps of a form of P450 from male bear liver microsomes. The specific content of the final preparation [designated P450(b-1)] was 11.26 nmol/mg protein, and recovery was 0.20% of the microsomal P450.

Figure 1 shows the result of SDS–PAGE at each purification step. Purified P450(b-1) showed a single band on SDS–PAGE (lane 5). Comparing the electrophoretic mobility of P450(b-1) with those of standard proteins, the apparent molecular weight was estimated to be 54,000. Absorption spectra of P450(b-1) are shown in Fig. 2. In the oxidized form, absorption maximum was observed at around 417 nm. A gentle shoulder was observed near 390 nm in this spectrum. These results indicate that 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 consistent with dog P450-D1 [6], horse P450(h-1) [8] and rat P450 UT-2(2C11) [5]. The carbon monoxide complex of the reduced form exhibited a Soret absorption peak at 450.5 nm.

Catalytic properties of liver microsomes and purified bear P450(b-1) in the reconstituted system. The content of P450 in the hepatic microsomes of bears was 0.31 nmol/mg protein (Table 1). Table 2 shows the drug-metabolizing activities of liver microsomes from rats and bears and purified rat P450 UT-2(2C11) and bear P450(b-1). Liver microsomes from bears possessed the highest

Table 2. Drug-metabolizing activities of liver microsomes from rats and bears and purified preparation of rat P450 UT-2(2C11) and bear P450(b-1) in a reconstituted system

Reactions	Microsomes*		Reconstituted system	
	Rat (nmol/min/mg protein)	Bear	Rat P450 UT-2 (nmol/min/nmol P450)	Bear P450(b-1)
Aminopyrine N-demethylation	3.14 ± 0.23	1.10 ± 0.04	8.64	13.62
Benzo[*a*]pyrene 3-hydroxylation	0.28 ± 0.04	0.17 ± 0.05	0.21	2.26
7-Ethoxycoumarin O-deethylation	0.69 ± 0.11	0.23 ± 0.07	1.37	0.64
Imipramine 2-hydroxylation	1.59 ± 0.06	0.49 ± 0.07	1.83	1.33
Imipramine N-demethylation	4.62 ± 0.13	0.91 ± 0.05	16.95	10.03
Propranolol 4-hydroxylation	1.58 ± 0.36	0.69 ± 0.11	1.19	0.52
Propranolol 5-hydroxylation	0.68 ± 0.21	1.47 ± 0.20	1.52	0.30
Propranolol 7-hydroxylation	0.44 ± 0.21	ND†	ND	ND

* Values are means ± SD of three (rats or bears) individual liver fractions.
† ND, not detectable.

activity in propranolol 5-hydroxylation, followed by aminopyrine N-demethylation, imipramine N-demethylation, propranolol 4-hydroxylation, imipramine 2-hydroxylation, 7-ethoxycoumarin O-deethylation and benzo[*a*]pyrene 3-hydroxylation. Propranolol 7-hydroxylase activity was absent in liver microsomes from bear. Rat liver microsomes showed the highest activity in imipramine N-demethylation, followed by aminopyrine N-demethylation, imipramine 2-hydroxylation, propranolol 4-hydroxylation, 7-ethoxycoumarin O-deethylation, propranolol 5-hydroxylation, propranolol 7-hydroxylation and benzo[*a*]pyrene 3-hydroxylation in that order. Therefore, the metabolic activity profiles of liver microsomes from bears and rats are clearly different. Except for propranolol 5-hydroxylase activity, monooxygenase activities in liver microsomes from bears were somewhat lower than those in rat liver microsomes. The reconstituted system containing P450(b-1) catalyzed aminopyrine N-demethylation and imipramine N-demethylation strongly among those reactions mentioned above. Comparing the activities of liver microsomes on the basis of P450, relatively higher activities of P450(b-1) were seen in aminopyrine N-demethylation and imipramine N-demethylation. In common with this, P450 UT-2(2C11) showed relatively higher activities in aminopyrine N-demethylation and imipramine N-demethylation. Antibody prepared against P450 2C11 has been known to inhibit imipramine N-demethylation up to 80% in male rats [24], indicating that imipramine N-demethylation is largely dependent on isozyme in liver microsomes of male rats. Thus, with minor exceptions, P450(b-1) and P450 UT-2(2C11) possess similar drug-metabolizing properties. P450(b-1) possessed relatively high benzo[*a*]pyrene 3-hydroxylase activity.

N-Terminal amino acid sequence and immunochemical properties of P450(b-1). Figure 3 shows the N-terminal amino acid sequence of P450(b-1) in comparison with the 2C subfamily. The amino acid sequences in the amino terminal region have been recognized as not being conserved among various forms of P450, but the N-terminal amino acid sequence of P450(b-1) is highly homologous to dog

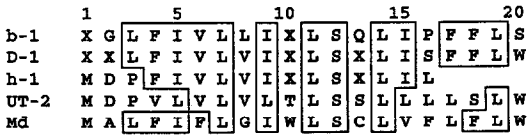

Fig. 3. Comparison of the N-terminal amino acid sequence of bear P450(b-1) with those of P450 2C members from different animal species. Data from dog P450 D-1, horse P450(h-1), rat P450 UT-2(CYP2C11), and rat P450 Md(CYP2C22) are from Komori *et al.* [6], Komori *et al.* [8], Funae and Imaoka [5], and Nagata *et al.* [25], respectively. The N-terminal amino acid sequence of purified P450(b-1) was determined by applying it (200 pmol) to an automatic protein sequencer. Amino acid residues identical to those of P450(b-1) are boxed. "X" indicates residues not determined.

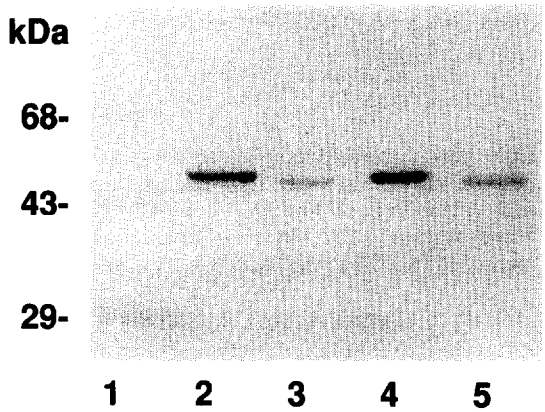

Fig. 4. Western blotting analysis of proteins cross-reactive with anti-P450 2C11 antibody. Western blotting-peroxidase staining analysis of proteins was carried out using anti-P450 UT-2(2C11) antibody. Lane 1, prestained standard protein (the same as those in Fig. 1); lane 2, 10 μg of rat liver microsomes; lane 3, 10 μg of bear liver microsomes; lane 4, 5 pmol of purified P450 UT-2(2C11); and lane 5, 5 pmol of purified P450(b-1).

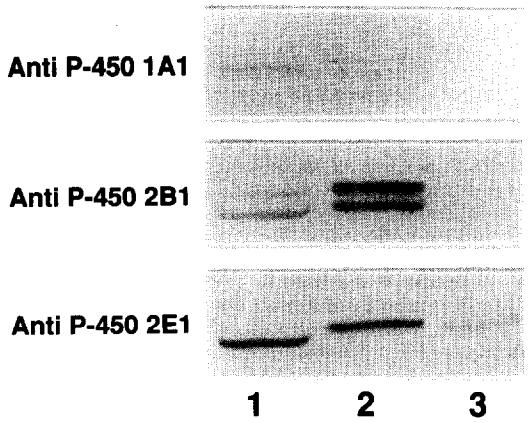

Fig. 5. Western blotting analyses of rat and bear liver microsomes and purified bear P450(b-1) using anti-P450 1A1, 2B1 and 2E1 antibodies. Lane 1, 10 μg of rat liver microsomes; lane 2, 10 μg of bear liver microsomes; and lane 3, 5 pmol of purified bear P450(b-1).

P450-D1 [6], horse P450(h-1) [8], rat P450 UT-2(2C11) [5], and rat P450 Md(2C22) [25], which are male-specific forms. It is noteworthy that the N-terminal amino acid sequence of P450(b-1) is the most highly homologous to dog P450-D1 (65%, 13 of 20 common residues) among other species. Both dogs and bears belong to *Canoidea* in the phylogenetic tree. The closeness of amino acid sequences corresponded well with the taxonomic relatedness. P450(b-1) from male brown bear liver microsomes was immunochemically cross-reactive with anti-P450 2C11 antibody (Fig. 4). Anti-P450 2C11 antibody strongly recognized epitopes of bear P450(b-1). Another immunochemically related P450 was found in bear hepatic microsomes with anti-P450 2C11 antibody. The apparent molecular weight of this P450 was larger than that of P450(b-1). Figure 5 shows Western blotting analysis of bear microsomes and bear P450(b-1) with anti-P450 1A1, 2B1 and 2E1 antibodies. Anti-P450 1A1 and 2B1 antibodies reacted with bear liver microsomes showing two very faint bands and three clear bands, respectively, but did not react with purified bear P450(b-1). Anti-P450 2E1 antibody faintly stained the purified bear P450. This is in agreement with the finding that antibodies prepared against the P450 2E subfamily have been known to recognize peptides that are also recognized by some anti-P450 2C antibodies [26]. But anti-P450 2E1 antibody clearly recognized only one band in bear liver microsomes. The apparent molecular weight of this peptide of bear liver microsomes was larger than that of P450(b-1).

In summary, all the catalytic and immunochemical properties and N-terminal amino acid sequences of P450(b-1) were similar to those of P450 2C11 and other P450s of the 2C subfamily. This suggests that P450(b-1) from bear liver microsomes belongs to the P450 2C subfamily.

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