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Purification from Liver Microsomes from Untreated Cynomolgus Monkeys of Cytochrome P450 Closely Related to Human Cytochrome P450 2B6

SHIGERU OHMORI, CHIZU SHIRAKAWA, KEIKO MOTOHASHI, HISAE YOSHIDA, HIROKO ABE, TOSHIHIRO NAKAMURA, TOHRU HORIE, HARUO KITAGAWA, KAZUO ASAOKA, TADAAKI RIKIHISA, YOSHIO KANAKUBO, and MITSUKAZU KITADA

Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan (S.O., C.S., K.M., H.Y., H.A., T.N., H.K.), Eisai Co. Ltd., 5-1-3 Tokodai, Tsukuba 330-26, Japan (T.H.), Primate Research Institute, Kyoto University, Kanbayashi, Inuyama 484, Japan (K.A.), and Hospital Pharmacy, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan (T.R., Y.K., M.K.).

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SUMMARY

A cytochrome P450 (P450) (referred to as P450CMLa) was purified and characterized from hepatic microsomes from untreated cynomolgus monkeys (*Macaca irus*). The final preparation was electrophoretically homogeneous and its estimated minimum molecular mass was 49.5 kDa. The amino-terminal amino acid sequence of the protein (first 34 residues) closely resembled that of the protein encoded by the 2B6 cDNA from humans (94%). This protein was cross-reactive with antibodies raised against P450 2B1 (P450 b), P450 2B11 (P450 PBD-2), and P450GP-1, which were purified from hepatic microsomes from phenobarbital-pretreated rats, beagle dogs, and guinea pigs, respectively. Also, the antibody raised against P450CMLa was able to cross-react with P450 2B1, P450 2B11, and P450GP-1. P450CMLa was capable of catalyzing benzphetamine *N*-demethylation and testosterone 16β-hydroxylation in a reconstituted system. Anti-P450CMLa antibody inhibited the activity of testosterone 16β-hydroxylase but not the activities of testosterone 2β- and 6β-hydroxylases in liver microsomes from cynomolgus monkeys. The content of P450CMLa, as estimated by immunoblot analysis, was 70 pmol/mg (about 5% of total P450). The protein immunoreactive with the anti-P450CMLa antibody was also present in liver microsomes from Japanese monkeys, baboons, common marmosets, and common squirrel monkeys. In liver microsomes from common squirrel monkeys, the content of protein immunoreactive with the anti-P450CMLa antibody and the activity of testosterone 16β-hydroxylase were effectively increased by pretreatment with phenobarbital. The antibody against P450CMLa strongly inhibited the activity of testosterone 16β-hydroxylase in liver microsomes not only from untreated cynomolgus monkeys but also from phenobarbital- and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys. These results indicated that the P450CMLa purified here is very similar to the forms of P450 classified into the 2B subfamily, in its amino-terminal amino acid sequence, catalytic activities, and immunochemical properties.

The P450s play an important role as the terminal oxidases in the hepatic monooxygenase system and can metabolize a variety of endogenous and exogenous substrates (1). To date, >150 P450 genes have been reported and classified into 27 families (2). Hepatic microsomal P450 isoforms were purified and characterized from a variety of animals, as well as from humans (3-6). It has been shown that substrate specificities and functions of P450s belonging to the same subfamily are somewhat different among animal species (7-9). Although nonhuman primates, which are genetically closer to humans than are rodents, would be a better source for a comparative study on drug metabolism, a limited number of studies on P450 purification carried out in nonhuman primates have been reported. After we first isolated a P450 from liver microsomes from untreated cynomolgus monkeys (10), several P450 forms were purified from liver microsomes from nonhuman primates (11-15). However, the 2B isoform, which is one of the phenobarbital-inducible forms in rodents (16-18), is still not well characterized in liver microsomes from primates. Although P450s were purified from liver microsomes from phenobarbital-pretreated marmosets (14) and baboons (15), the purified marmoset P450 was not well characterized and the baboon P450s (FA and FI) purified were closely related to the 2A and 3A subfamilies, respectively, rather than the 2B subfamily. On the other hand, the sequence of the human 2B6 gene has been reported, but the function of the gene product has not been well characterized (19).

In this paper, we describe the purification and characterization of a new form of P450 from untreated cynomolgus monkeys that seems to be classified into the 2B subfamily. In addition,

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ABBREVIATIONS: P450, cytochrome P450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the expression of P450 cross-reactive with antibodies to the P450s purified from liver microsomes from other nonhuman primates and the inducibility of the P450 by phenobarbital pretreatment were also investigated.

Experimental Procedures

Materials. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Octamethylenediamine, 7-ethoxycoumarin, and 7-hydroxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Silica columns and L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine were purchased from Sensyu Scientific Co. (Tokyo, Japan) and Serdary Research Laboratories (Ontario, Canada), respectively. Testosterone and cholic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 16β-Hydroxylated testosterone was obtained from Steraloids Co. (Wilton, NH), and other metabolites of testosterone were generous gifts from Dr. Kirk, Queen Mary College, University of London. Benzphetamine hydrochloride and pregnenolone 16α-carbonitrile were kindly provided by Upjohn Pharmaceutical Ltd. (Tokyo, Japan). Emulgens 911 and 913 were kindly provided by Kao Atlas Co. (Tokyo, Japan). NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The reagents for gel electrophoresis and immunoblotting were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of reagent or analytical grade.

Animals, pretreatment, and preparation of microsomes. Cynomolgus monkeys (male, 3.5–4.1 kg) and common squirrel monkeys (male, 750–1000 g) were purchased from Kasho Co. Ltd. (Tokyo, Japan). Doguera baboons (male, 22–30 kg) were purchased from Japan Monkey Center (Inuyama, Japan). Common marmosets (male, 300–410 g; female, 360–380 g) were provided by Pharmaceutical Research Center, Meiji Seika Kaisha Ltd. (Yokohama, Japan). Liver samples from Japanese monkeys (male, 5.6–9.5 kg) were provided by Primate Research Institute, Kyoto University (Inuyama, Japan). Common squirrel monkeys were pretreated with phenobarbital (20 mg/kg) or pregnenolone 16α-carbonitrile (40 mg/kg), intraperitoneally, for 3 days before being killed. Hartley guinea pigs (300–500 g), Sprague-Dawley rats (120–150 g), and Japan White rabbits (2.5–3.0 kg) were purchased from Takasugi Experimental Animals Co. (Kasukabe, Japan). Beagle dogs (10–15 kg) were kindly provided by Eisai Co. (Tsukuba, Japan). Rats and guinea pigs were pretreated with 0.1% (w/v) sodium phenobarbital in drinking water for 7 days before being killed. The phenobarbital treatment for beagle dogs followed the schedule of Duignan *et al.* (6). Liver microsomes were prepared by differential centrifugation as described previously (10, 20). The microsomal pellets were finally suspended in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA. The microsomal suspensions were stored at −80° until used.

Purification of P450 from liver microsomes from untreated cynomolgus monkeys. Microsomes (approximately 820 mg of protein, 1.2 nmol of P450/mg) were solubilized in buffer A [100 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.6 μM phenylmethylsulfonyl fluoride] containing 0.6% sodium cholate. Solubilized supernatants were loaded onto a *n*-octylamino-Sepharose 4B column (2.5 × 25 cm) equilibrated with buffer A. P450 was eluted with buffer A containing 0.4% sodium cholate and 0.08% Emulgen 913. SDS-PAGE and Western blot analysis with anti-P450b antibody were routinely carried out with each fraction before the individual fractions were pooled. The pooled fraction was diluted 3-fold with 20% (v/v) glycerol and applied to a hydroxylapatite column (2.5 × 12 cm) equilibrated with 33 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol and 0.2% Emulgen 913. The fractions containing P450 that had been passed through the column were pooled, diluted 2-fold with 20% (v/v) glycerol, and applied to a second hydroxylapatite column (1.6 × 7 cm; Bio-Rad Laboratories, Richmond, CA). After the fractions containing P450, which was recovered by washing the column with 80 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol and 0.2% Emulgen 913, were pooled and dialyzed against 5 mM buffer B [5 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1% sodium cholate, and 0.2% Emulgen 913], the pooled fractions were applied to a DE-52 column (2 × 13 cm; Whatman BioSystems Ltd., Maidstone, England) equilibrated with 5 mM buffer B. The DE-52 column was then washed with 120 ml each of 5 mM buffer B and 10 mM buffer B. When a few contaminant proteins still remained in the fractions eluted from the DE-52 column, immunoaffinity column (0.5 × 5 cm) chromatography was carried out according to the method reported previously (21). Excess amounts of nonionic detergent in the purified preparation were removed by adsorption of protein onto an hydroxylapatite column (0.5 × 2 cm). After the column was washed with 10 mM potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol until optical density at 280 nm was reduced to <0.01, the P450 (referred to as P450CMLa) was eluted by increasing the concentration of potassium phosphate buffer to 300 mM.

Purification of other enzymes. P450b, P450PBD-2, and P450GP-1 were purified from liver microsomes from phenobarbital-pretreated rats, beagle dogs, and guinea pigs, respectively, according to the methods described elsewhere (21, 6, 9). Three forms of P450 purified from rats, beagle dogs, and guinea pigs were identified as P450 2B1, P450 2B11, and P450GP-1, respectively, by determination of the amino-terminal amino acid sequences of the purified P450s. The sequences of 25 amino-terminal residues of P450b, P450PBD-2, and P450GP-1 purified in this study were identical to those of P450s reported previously (6, 9, 22). Cytochrome b_5 was purified from rat liver microsomes by a combination of the methods of Spatz and Strittmatter (23) and Ito (24). The specific contents of purified P450 2B1, P450 2B11, P450GP-1, and cytochrome b_5 were 13.6, 19.5, 12.9, and 33.1 nmol/mg of protein, respectively. NADPH-cytochrome *c* (P450) reductase was purified from liver microsomes from phenobarbital-pretreated rats by the method of Yasukochi and Masters (25), with minor modifications. The specific activity of purified reductase was 45.0 units/mg of protein. One unit of reductase activity was defined as the amount of the enzyme catalyzing the reduction of 1 μmol of cytochrome *c*/min (26)

Assay for monooxygenase activities. A typical reaction mixture for the microsomal system consisted of 100 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, substrate, microsomes (testosterone hydroxylase, 0.2 mg of protein; *N*-demethylase, 1 mg of protein), and an NADPH-generating system (0.33 mM NADP, 0.1 unit of glucose-6-phosphate dehydrogenase, 8 mM glucose-6-phosphate, and 6 mM $MgCl_2$), in a final volume of 1 ml. A typical reaction mixture for the reconstituted system consisted of 0.1 nmol of P450, 1 unit of NADPH-cytochrome *c* (P450) reductase, 25 μg of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 100 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, substrate, and the NADPH-generating system, in a final volume of 1 ml. Aniline (5 mM), *N*,*N*-dimethylaniline (5 mM), ethylmorphine (5 mM), benzphetamine (1 mM), *p*-nitroanisole (0.5 mM), 7-ethoxycoumarin (0.2 mM), and testosterone (0.2 mM) were used as the substrates. The reaction was initiated by the addition of the NADPH-generating system and was carried out for 10 min (testosterone hydroxylase and 7-ethoxycoumarin *O*-deethylase) or 15 min (other assays) at 37° with shaking, under aerobic conditions. The testosterone hydroxylase activity was measured by the method of Hayashi *et al.* (27) with minor modifications. *N*-Demethylation of benzphetamine, *N*,*N*-dimethylaniline, and ethylmorphine was assayed by measuring the amount of liberated formaldehyde, using the Nash reagent (28). The activities of aniline *p*-hydroxylase and *p*-nitroanisole *O*-demethylase were measured by the methods of Imai *et al.* (29) and Kamataki *et al.* (30), respectively. 7-Ethoxycoumarin *O*-deethylase activity was measured fluorometrically by the method of Greenlee and Poland (31).

Other methods. Antibodies were raised against P450CMLa, P450GP-1, P450 2B1, and P450 2B11 in Japan White rabbits. The sensitization schedule and procedures for IgG preparation were as described elsewhere (32). SDS-PAGE and Western blot peroxidase-antiperoxidase staining were carried out essentially according to the

methods of Laemmli (33) and Guengerich *et al.* (34), respectively. 3,3′-Diaminobenzidine was used as the substrate for peroxidase. Intensities of stained bands were measured by absorbance at 400 nm, with the use of a Shimazu CS910 dual-wavelength thin layer chromatography scanner equipped with an integrator. Purified P450CMLa was used as the standard. Total P450 content was measured by the method of Omura and Sato (35), in the presence of 20% (v/v) glycerol and 0.2% Emulgen 911. Protein was determined as described by Lowry *et al.* (36), using bovine serum albumin as the standard. Significance was analyzed by Student's *t* test.

Results

Purification of P450CMLa from hepatic microsomes from untreated cynomolgus monkeys. P450CMLa was purified from hepatic microsomes from untreated monkeys as described in Experimental Procedures. P450CMLa did not adsorb to the first hydroxylapatite column under the condition of 33 mM potassium phosphate buffer, pH 7.25, and approximately 8% of the P450 applied was recovered in a pass-through fraction. Therefore, most of the P450 isoforms were able to be separated from P450CMLa during this column step. In the DE-52 column chromatography step P450CMLa was sometimes unable to be well separated from other proteins (probably most of which were epoxide hydrolases). Because the majority of these contaminant proteins were eluted from the DE-52 column before P450CMLa, antibodies were produced against contaminating protein using these fractions as antigens, and an immunoaffinity column that immobilized these antibodies was prepared. P450CMLa passed through the immunoaffinity column and contaminating proteins were retained. This column step was repeated until contaminating proteins had been eliminated from P450CMLa-rich fractions, as seen in SDS-PAGE. Specific content and recovery of final preparations were 11.8–14.1 nmol/mg and 0.2–0.4%, respectively. The purified P450CMLa was apparently homogeneous, as judged by SDS-PAGE (Fig. 1), and the apparent molecular mass of P450CMLa was estimated to be 49.5 kDa when phospholylase *b*, albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor were used as the standard proteins.

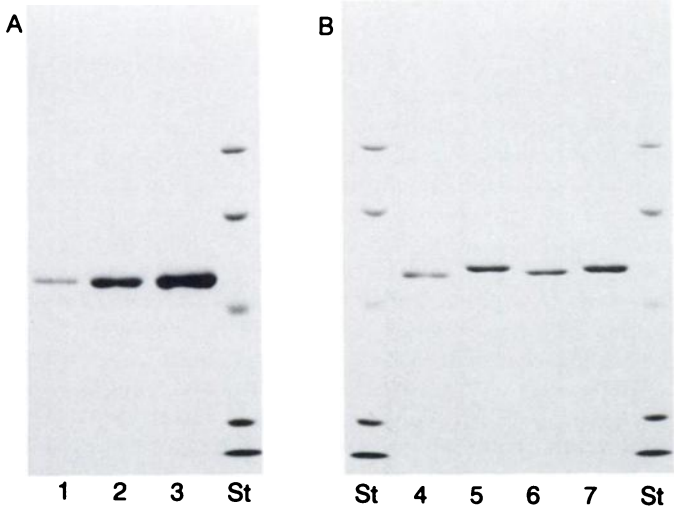

Fig. 1. SDS-PAGE of purified P450CMLa, P450 2B1, P450 2B11, and P450GP-1. The purified preparation of each cytochrome was electrophoresed by the method of Laemmli (33). *St* (standard protein molecular mass makers), phospholylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa). A, *Lanes 1-3* contained 10, 20, and 40 pmol of P450CMLa, respectively. B, *Lanes 4* (P450CMLa), *5* (P450b), *6* (P450PBD-2), and *7* (P450GP-1) contained 10 pmol each of P450 preparation.

P450CMLa was found to have an α-band at 567–568 nm, a β-band at 534–536 nm, and a Soret peak at 417 nm in the oxidized form, suggesting that the P450 was in a low spin state. The carbon monoxide-bound reduced form of P450CMLa showed a Soret peak at 450–451 nm (data not shown).

Amino-terminal amino acid sequence. The purified P450CMLa was subjected to automated Edman degradation for estimation of amino-terminal sequence. The purity of this enzyme was confirmed by the presence of a single amino-terminal sequence with no unidentified residues. When the sequence of P450CMLa was compared with those of monkey P450s reported previously, it was obvious that P450CMLa has a different amino-terminal amino acid sequence from those of P450MK1, P450MK2, and P450FI (Fig. 2). In contrast, the sequence of the first 34 amino-terminal amino acids of P450CMLa was similar to those of P450s belonging to the 2B subfamily (Fig. 2). Interestingly, the deduced amino-terminal amino acid sequence of human 2B6 (19) was identical to that of P450CMLa except for two residues. The order of similarity of the amino-terminal amino acid sequences to that of P450CMLa was as follows: 2B6 (human) > 2B11 (dog) > 2B4 (rabbit) > 2B1 (rat) = 2B2 (rat) = 2b-10 (mouse) > 2B3 (rat).

Immunochemical properties of purified enzymes. Using antibodies raised against P450CMLa, P450 2B1, P450 2B11, and P450GP-1, the immunochemical similarities among these P450s were examined by immunoblot analysis (Fig. 3). The anti-P450CMLa antibody could cross-react not only with the antigen but also with P450 2B1, P450 2B11, and P450GP-1. In addition, the anti-P450 2B1, -P450 2B11, and -P450GP-1 antibodies were able to cross-react not only with corresponding antigens but also with other P450s used here. The antibodies raised against monkey P450 that had been purified in our laboratory (10) and rat P450 1A1 and 1A2 did not show any cross-reactivity with P450CMLa (data not shown).

Catalytic activities of P450CMLa. Table 1 shows the catalytic properties of P450CMLa. P450CMLa had high demethylase activities towards benzphetamine and aminopyrine. On the other hand, the P450 showed a low ability to metabolize 7-ethoxycoumarin. Interestingly, testosterone 16β-hydroxylation, which was one of the reactions catalyzed specifically by P450 2B1 (42) and P450GP-1 (9), was also effectively catalyzed by P450CMLa. The ratio of 16β-hydroxytestosterone to 16α-hydroxytestosterone produced by P450CMLa was 145. The activities of 7-ethoxycoumarin *O*-deethylase and testosterone 16α- and 16β-hydroxylase were enhanced 100, 140, and 40%, respectively (Table 1), when cytochrome b_5 purified from rat liver microsomes was added to the reconstituted system at equimolar ratio to P450. Hydroxylase activities for testosterone at the 2β-, 6β-, 16α-, and 16β-positions in hepatic microsomes from cynomolgus monkeys were 0.809 ± 0.059, 5.383 ± 0.341, 0.031 ± 0.004, and 0.207 ± 0.014 nmol/mg/min, respectively (five experiments). No sex difference was observed in the activities of testosterone 2β-, 6β-, 16α-, and 16β-hydroxylases in hepatic microsomes from cynomolgus monkeys (data not shown).

Immunoinhibition of the activity of testosterone 16β-hydroxylase by the anti-P450CMLa antibody in liver microsomes from untreated cynomolgus monkeys. The

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                    1   1   2   2   3
                5   0   5   0   5   0
P450CMLa        MELSVLLFLALLTGLLLLLVQRHPNAHGRLPPGP
P450MK1         MDSLVVLVLXLSXLLLLSLWRQ              (45 %)
P450MK2         MDLIPDLAVETWLLLAVTLVLL               (27 %)
P450FI          MLASGLLLVALLAXLTVMVL                 (37 %)

P450CMLa        MELSVLLFLALLTGLLLLLVQRHPNAHGRLPPGP
P450 2B6        MELSVLLFLALLTGLLLLLVQRHPNTHDRLPPGP   (94 %)
hIIB3           MELSVLLFLALLTGLLLLLVQRHPNSHGTLPPGP   (94 %)
P450 2B11       MELSVLLLLALLTGLLLLMARGHPKAHGRLPPGP   (82 %)
P450 2B4        MEFSLLLLLAFLAGLLLLLFRGHPKAHGRLPPGP   (74 %)
P450 2B1        MEPSILLLLALLVGFLLLLVRGHPKSRGNFPPGP   (65 %)
P450 2B2        MEPSILLLLALLVGFLLLLVRGHPKSRGNFPPGP   (65 %)
P450 2b-10      MEPSVLLLLALLVGFLLLLARGHPKSRGNFPPGP   (65 %)
P450 2B3        MDTSVLLLLAVLLSFLLFLVRGHAKVHGHLPPGP   (62 %)
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Fig. 2. Comparison of the amino-terminal amino acid sequence of P450CMLa with those of monkey P450 isoforms and of the 2B subfamily. Purified P450CMLa (1 nmol) was subjected to automated amino-terminal amino acid sequence analysis. Yields of M, E, L, S, V, L, L, F, L, A, L, L, T, G, L, L, L, L, L, V, Q, R, H, P, N, A, H, G, R, L, P, P, G, and P were 836, 643, 788, 100, 542, 608, 534, 528, 651, 413, 603, 659, 165, 306, 547, 583, 580, 585, 604, 353, 213, 220, 121, 355, 192, 287, 109, 226, 193, 332, 327, 344, 226, and 321 pmol, respectively. The sequences for P450MK1 (12), P450MK2 (13), P450FI (15), P450 2B6 (19), hIIB3 (19), P450 2B11 (37), P450 2B4 (38), P450 2B1 (22), P450 2B2 (39), P450 2b-10 (40), and P450 2B3 (41) are given for comparison, and amino acids represented as *outlined letters* are different from the sequence of P450CMLa. *X*, Unidentified amino acid. *Numbers in parentheses*, percentages of similarity with the sequence of P450CMLa.

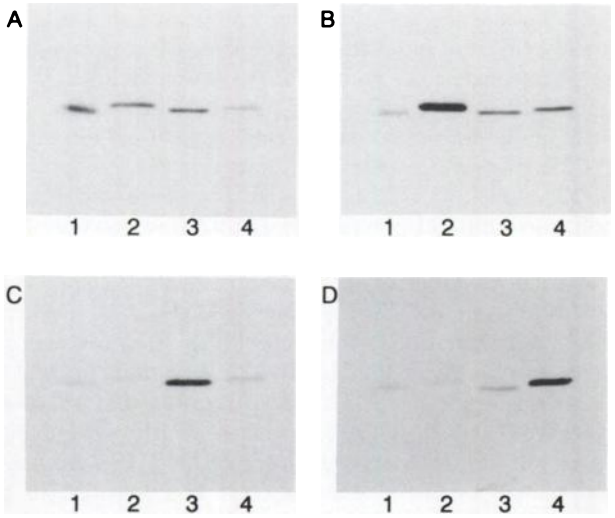

Fig. 3. Immunoblot analysis of purified P450s. Six hundred femtomoles each of P450CMLa (*lane 1*), P450 2B1 (*lane 2*), P450 2B11 (*lane 3*), and P450GP-1 (*lane 4*) were electrophoresed by the method of Laemmli (33) and analyzed for cross-reactivity with anti-P450CMLa (A), -P450 2B1 (B), -P450 2B11 (C), and -P450GP-1 (D) antibodies by the method of Guengerich *et al.* (34).

TABLE 1

Catalytic properties of purified P450CMLa

Numbers in parentheses represent the activity measured in the presence of cytochrome b_5. Assay methods were described in Experimental Procedures.

Substrate	Reaction	Activity
		nmol/nmol of P450/min
Benzphetamine	*N*-Demethylation	63
Aminopyrine	*N*-Demethylation	42
Ethylmorphine	*N*-Demethylation	36
N,N-Dimethylaniline	*N*-Demethylation	20
Aniline	*p*-Hydroxylation	4.1
7-Ethoxycoumarin	*O*-Deethylation	0.97 (2.0)
Testosterone	16α-Hydroxylation	0.07 (0.17)
	16β-Hydroxylation	10 (14)

results described above indicated the possible involvement of P450CMLa in testosterone 16β-hydroxylation in liver microsomes from cynomolgus monkeys. As shown in Fig. 4, anti-P450CMLa IgG inhibited >70% of the 16β-hydroxylation of testosterone, indicating that P450CMLa is one of the major

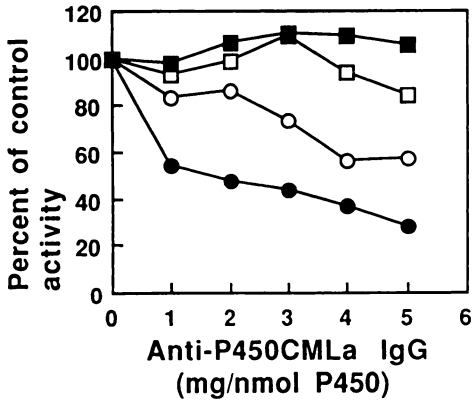

Fig. 4. Immunoinhibition of testosterone hydroxylases with the anti-P450 antibody in hepatic microsomes of untreated cynomolgus monkeys. Anti-P450CMLa IgG and control rabbit IgG were added in various ratios to maintain a total of 1 mg of protein. Control activities of 2β (□)-, 6β (■)-, 16α (○)-, and 16β (●)-hydroxylases were 0.924, 6.326, 0.050, and 0.284 nmol/mg/min, respectively.

forms of P450 that contribute to this reaction in hepatic microsomes from untreated cynomolgus monkeys. Anti-P450CMLa IgG also exerted an inhibitory effect on the 16α-hydroxylation of testosterone (43% inhibition at the concentration of 5 mg of IgG/nmol of P450). The results showing that the activities of testosterone 2β- and 6β-hydroxylations were not inhibited by anti-P450CMLa IgG were in accordance with the finding that P450CMLa did not catalyze 2β- and 6β-hydroxylations of testosterone. In addition, anti-P450CMLa IgG inhibited by 40% the demethylation of benzphetamine in liver microsomes from untreated cynomolgus monkeys (data not shown).

Estimation of the protein that is cross-reactive with the anti-P450CMLa antibody in liver microsomes from nonhuman primates. Because it has been reported that P450 content, and the capabilities for drug metabolism, in hepatic microsomes are somewhat different among the nonhuman primates (43), we examined whether the protein that is immunoreactive with the anti-P450CMLa antibody is present in liver microsomes from baboons, Japanese monkeys, and marmosets. The total P450 contents in liver microsomes from cynomolgus monkeys and Japanese monkeys were higher than in those from baboons and marmosets (Table 2). As shown in Fig. 5, the protein immunoreactive with the anti-P450CMLa antibody

TABLE 2

Contents of protein immunoreactive with the anti-P450CMLa antibody in hepatic microsomes from nonhuman primates

Each value represents mean ± standard error.

	Total P450 content	Protein immunoreactive with anti-P450CMLa antibody[a]
	nmol/mg	*pmol or unit/mg*
Cynomolgus monkey (n = 4)	1.41 ± 0.14	71 ± 8
Baboon (n = 3)	0.65 ± 0.11	31 ± 3
Japanese monkey (n = 5)	1.33 ± 0.04	84 ± 12
Common marmoset		
Male (n = 4)	0.62 ± 0.03	21 ± 1
Female (n = 4)	0.65 ± 0.10	15 ± 4

[a] Same staining intensity as 1 pmol of P450CMLa was defined as 1 unit of immunoreactive protein.

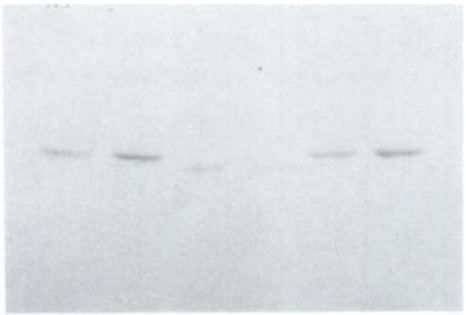

Fig. 5. Immunoblot analysis of hepatic microsomes from nonhuman primates. *Lane 1*, baboon (5 pmol of P450); *lane 2*, Japanese monkey (5 pmol of P450); *lane 3*, male marmoset (5 pmol of P450); *lane 4*, female marmoset (5 pmol of P450); *lane 5*, cynomolgus monkey (5 pmol of P450); *lane 6*, purified P450CMLa (0.22 pmol).

was detectable in all of the microsomes used. The immunoreactive proteins in liver microsomes from cynomolgus monkey, baboon, and Japanese monkey had apparently the same relative mobilities as P450CMLa, whereas the mobilities on SDS-PAGE of proteins immunoreactive with the anti-P450CMLa antibody in male and female marmoset were slightly greater than that of P450CMLa (Fig. 5). In hepatic microsomes from cynomolgus monkeys, the amount of P450CMLa was 70 pmol/mg (about 5% of total P450), indicating that the P450 isoform is constitutively expressed but not a major form in cynomolgus monkey livers (Table 2). Although it was impossible to quantitate the amounts of protein immunoreactive with the antibody in liver microsomes from other animal species, we tried to semiquantify the protein that is immunoreactive with the anti-P450CMLa antibody in liver microsomes from baboons, Japanese monkeys, and marmosets (Table 2). The staining intensity obtained with 1 pmol of P450CMLa was defined as 1 unit of immunoreactive protein. The amounts of the protein immunoreactive with the anti-P450CMLa antibody in hepatic microsomes from baboons, Japanese monkeys, and marmosets were 31, 83, and 21 units/mg, respectively. There were no sex differences in the amounts of total P450 or the protein immunoreactive with the anti-P450CMLa antibody in hepatic microsomes from marmosets.

Induction of protein that is immunoreactive with the anti-P450CMLa antibody in common squirrel monkeys. To clarify the inducibility of this protein, we examined the effects of phenobarbital and pregnenolone 16α-carbonitrile pretreatment on the monooxygenase system and the protein immunoreactive with the anti-P450CMLa antibody in liver microsomes from common squirrel monkeys. The activities of testosterone 16β-hydroxylase and benzphetamine N-demethylase were increased by the phenobarbital pretreatment 13- and 5-fold, respectively, compared with control (Table 3). The activities of ethylmorphine N-demethylase and testosterone 2β-, 6β-, and 16α-hydroxylases were also increased by the treatment with phenobarbital. Pregnenolone 16α-carbonitrile pretreatment resulted in a significant increase in the activities of testosterone 2β- and 6β-hydroxylation and ethylmorphine N-demethylation, which are specific reactions catalyzed by 3A isoforms in rat liver microsomes (3). The activity of testosterone 16α-hydroxylase was increased up to 10 times the control value, whereas that of testosterone 16β-hydroxylase was only slightly increased by treatment of common squirrel monkeys with pregnenolone 16α-carbonitrile.

As in the case of baboons and Japanese monkeys (Fig. 5), the anti-P450CMLa antibody recognized a protein that shows the same relative mobility as P450CMLa on SDS-PAGE of liver microsomes from untreated and phenobarbital- and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys (Fig. 6). As shown in Table 4, relative amounts of the protein cross-reactive with the anti-P450CMLa antibody in liver microsomes from control and phenobarbital-pretreated monkeys were 5 and 280 units/mg, respectively. This protein that is immunochemically related to P450CMLa was not induced by pregnenolone 16α-carbonitrile pretreatment (Table 4).

Fig. 7 shows the effects of anti-P450CMLa and -P450 3A4 IgGs on testosterone hydroxylation in liver microsomes from phenobarbital- and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys. The activity of testosterone 16β-hydroxylase in liver microsomes from phenobarbital-pretreated common squirrel monkeys was strongly inhibited by anti-P450CMLa IgG but not by anti-P450 3A4 IgG. In contrast, the activities of testosterone 2β- and 6β-hydroxylases in hepatic microsomes from phenobarbital- and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys were inhibited by anti-P450 3A4 IgG but not by anti-P450CMLa IgG. The activity of testosterone 16α-hydroxylase was more sensitive to anti-P450CMLa IgG in phenobarbital-pretreated animals than in pregnenolone 16α-carbonitrile-pretreated animals.

Discussion

Although P450 isoforms classified into the 2B subfamily have been well characterized in rodents (3–5), attempts at purification and characterization of P450s belonging to this subfamily have been unsuccessful in primates. In this report, we purified and characterized P450 (P450CMLa) from hepatic microsomes from untreated cynomolgus monkeys. The purified P450CMLa yielded a single amino-terminal amino acid sequence without unidentified residues, indicating the purity of this protein. The amino-terminal amino acid sequence of P450CMLa showed 62–94% identity to those of P450s belonging to the 2B subfamily (19, 22, 37–41). The sequence of P450CMLa is most similar to that of human 2B6. When the 34 amino-terminal residues of P450CMLa were compared with those of human 2B6, the changes in amino acid residues between them were found only at the 26- and 28-positions. It has been shown that a 2B6 expressed in a vaccinia virus system had a reduced CO-binding

TABLE 3

Effects of phenobarbital and pregnenolone 16α-carbonitrile pretreatment on the metabolism of benzphetamine, ethylmorphine, and testosterone in hepatic microsomes from common squirrel monkeys

Each value represents mean ± standard error from three samples.

	Activity					
	Benzphetamine N-demethylase	Ethylmorphine N-demethylase	Testosterone 2β-hydroxylase	Testosterone 6β-hydroxylase	Testosterone 16α-hydroxylase	Testosterone 16β-hydroxylase
	nmol/mg/min					
Control	1.56 ± 0.51	3.49 ± 0.45	0.202 ± 0.028	2.34 ± 0.36	0.013 ± 0.001	0.036 ± 0.001
Phenobarbital	7.92 ± 0.47[a]	18.62 ± 0.39[a]	0.931 ± 0.059[a]	10.40 ± 0.53[a]	0.143 ± 0.020[b]	0.484 ± 0.042[c]
Pregnenolone 16α-carbonitrile	1.71 ± 0.25	5.71 ± 0.61[b]	0.337 ± 0.023[c]	3.91 ± 0.31[c]	0.130 ± 0.021[b]	0.046 ± 0.003[b]

[a] Significantly different from control, $p < 0.001$.
[b] $p < 0.05$.
[c] $p < 0.01$.

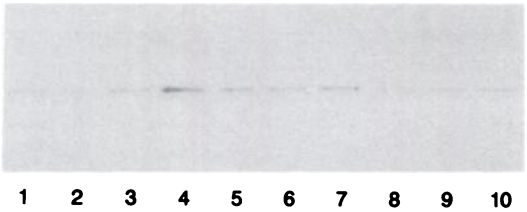

Fig. 6. Immunoblot analysis of liver microsomes from control, phenobarbital-pretreated, and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys. *Lanes 1-3*, control microsomal protein containing 5 pmol of P450; *lane 4*, purified P450CMLa (0.2 pmol); *lanes 5-7*, phenobarbital-pretreated microsomal protein containing 0.5 pmol of P450; *lanes 8-10*, pregnenolone 16α-carbonitrile-pretreated microsomal protein containing 5 pmol of P450.

TABLE 4

Effect of phenobarbital and pregnenolone 16α-carbonitrile pretreatment on the content of protein immunoreactive with the anti-P450CMLa antibody in hepatic microsomes from common squirrel monkeys

	Total P450 content	Protein immunoreactive with anti-P450CMLa antibody[a]
	nmol/mg	unit/mg
Control	0.30 ± 0.02	5.0 ± 0.2
Phenobarbital	1.65 ± 0.19[b]	279.7 ± 17.2[c]
Pregnenolone 16α-carbonitrile	0.56 ± 0.04[c]	6.1 ± 0.6

[a] Same staining intensity as 1 pmol of P450CMLa was defined as 1 unit of immunoreactive protein.
[b] Significantly different from control, $p < 0.05$.
[c] $p < 0.01$.

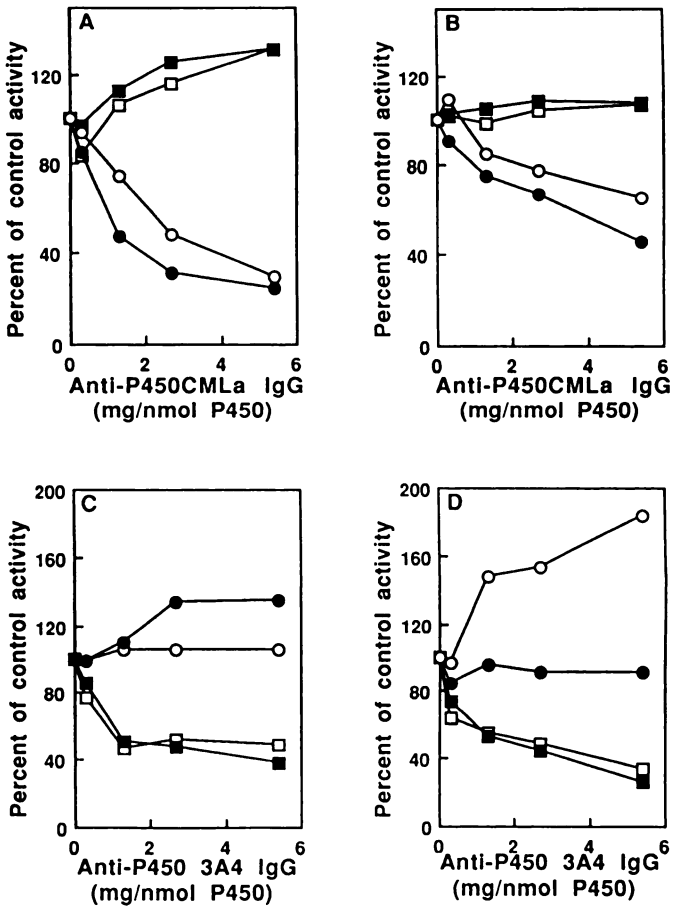

Fig. 7. Immunoinhibition of testosterone hydroxylases in hepatic microsomes of phenobarbital- and pregnenolone 16α-carbonitrile-pretreated common squirrel monkeys. Anti-P450CMLa IgG (A and B) or anti-P450 3A4 IgG (C and D) and control rabbit IgG were added in various ratios to maintain a total of 1.25 mg of protein. A and C, Control activities of 2β (□)-, 6β (■)-, 16α (○)-, and 16β (●)-hydroxylases in hepatic microsomes from phenobarbital-pretreated animals were 0.964, 10.984, 0.114, and 0.354 nmol/mg/min, respectively. B and D, Control activities of 2β (□)-, 6β (■)-, 16α (○)-, and 16β (●)-hydroxylases in hepatic microsomes from pregnenolone 16α-carbonitrile-pretreated animals were 0.313, 3.506, 0.109, and 0.039 nmol/mg/min, respectively.

spectrum with an absorption maximum of 452 nm and was able to catalyze 7-ethoxycoumarin *O*-deethylation (19). However, because catalytic activities of the expressed 2B6 other than 7-ethoxycoumarin *O*-deethylation were not measured, data presently available are not sufficient for comparison of catalytic properties between P450CMLa and human 2B6. Not only the amino-terminal amino acid sequence but also the immunochemical properties of P450CMLa were closely related to those of 2B isoforms. However, the precipitation line of P450CMLa with the anti-P450CMLa antibody clearly spurred with those of P450 2B1, P4502B11, and P450GP-1 with the anti-P450CMLa antibody in Ouchterlony double-diffusion analysis, indicating that antigenic sites of P450CMLa are not the same as those of P450 2B1, P450 2B11, and P450GP-1 (data not shown). The amount of P450CMLa immunochemically determined was about 5% of total P450, indicating that P450CMLa is a minor form of P450 in liver microsomes from cynomolgus monkeys.

It has been suggested that the monkey is a good animal model for human polymorphism of the 2D subfamily (44). Therefore, we examined the possibility of the presence of genetic poly-

morphism for the protein immunoreactive with the anti-P450CMLa antibody in Japanese monkeys. In the liver microsomes from these Japanese monkeys, we found the protein immunoreactive with the anti-P450CMLa antibody in all of the samples used ($n = 20$) (data not shown). These results and other results (Figs. 5 and 6; Tables 2 and 4) indicated that the protein immunoreactive with the anti-P450CMLa antibody is constitutively expressed in nonhuman primates.

The activity of testosterone 16α-hydroxylation was induced by both phenobarbital and pregnenolone 16α-carbonitrile treatment. These results and the results from immunoinhibition studies indicated the possibility of the presence of multiple isoforms able to hydroxylate testosterone at the 16α-position in hepatic microsomes from nonhuman primates, as in the case of rats, in which both P450 2B1 and P450 2C11 are known to hydroxylate testosterone at the 16α-position (3).

Similar to the other 2B isoforms, P450CMLa has high catalytic activities for benzphetamine and aminopyrine *N*-demethylations. In addition, P450CMLa has a high catalytic activity for testosterone 16β-hydroxylation (Table 1). It has been reported that testosterone 16β-hydroxylation is one of the reactions specifically catalyzed by P450 2B1 in rats (42), and the ratio of 16β-hydroxytestosterone to 16α-hydroxytestosterone is different between P450 2B1 and P450GP-1 (9). The ratio of 16β-hydroxytestosterone to 16α-hydroxytestosterone formation catalyzed by P450CMLa is extremely high, compared with the values reported for other 2B isoforms (9, 42), indicating a large stereoselectivity of P450CMLa for testosterone 16β-hydroxylation. Induction studies clearly revealed that the protein that is immunoreactive with the anti-P450CMLa antibody is one of the phenobarbital-inducible forms and the induced protein acts as a testosterone 16β-hydroxylase in hepatic microsomes from nonhuman primates (Figs. 6 and 7; Table 4).

Uno and Imai (45) have recently demonstrated that the amino acids between positions 90 and 125 from the amino terminus of 2C2 and 28 residues of the carboxyl terminus of 2C14 were very important for testosterone 16β-hydroxylation, using chimeric rabbit P450. Therefore, analysis of the structure will be required for an understanding of the stereoselectivity of P450CMLa for testosterone hydroxylation.

In summary, we have purified a P450, P450CMLa, related to the 2B gene subfamily from liver microsomes from untreated cynomolgus monkeys. Purified P450CMLa was highly similar to human 2B6 in amino-terminal amino acid sequence. Because human 2B6 is still not well characterized, this is the first report on the purification and characterization of a P450 belonging to the 2B subfamily in primate liver microsomes. P450CMLa acts as a testosterone 16β-hydroxylase in hepatic microsomes and shows high activity for *N*-demethylation of benzphetamine and aminopyrine. P450CMLa is expressed constitutively as a minor form of P450 in liver microsomes from cynomolgus monkeys and is one of the forms inducible by phenobarbital in nonhuman primates.

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Send reprint requests to: S. Ohmori, Ph.D., Laboratory of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan.