J. Biochem. **139**, 865–872 (2006)
doi:10.1093/jb/mvj093

Cynomolgus Monkey Cytochrome P450 2C43: cDNA Cloning, Heterologous Expression, Purification and Characterization

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Received December 2, 2005; accepted March 13, 2006

The cDNA of cytochrome P450 (CYP) 2C43 was cloned from cynomolgus monkey liver by RT-PCR. The deduced amino acid sequence showed 93% and 91% identity to human CYP2C9 and CYP2C19, respectively. The cDNA was expressed in *Escherichia coli* and purified by a series of chromatography steps, yielding a specific content of 11.5 nmol P450/mg protein. The substrate specificity of the purified CYP2C43 was examined in a reconstitution system comprising NADPH-P450 reductase, lipid, cytochrome b_5 and CYP2C marker substrates. The purified CYP2C43 showed high activity for testosterone 17-oxidation and progesterone 21-hydroxylation, which were also observed for CYP2C19 but not CYP2C9. In addition, CYP2C43 showed activity for (*S*)-mephenytoin 4′-hydroxylation, a marker reaction for CYP2C19. With CYP2C9 marker substrates, CYP2C43 exhibited low activity for diclofenac 4′-hydroxylation and no activity for tolbutamide *p*-methylhydroxylation. Therefore, in terms of substrate specificity, our results indicate that CYP2C43 is similar to CYP2C19, rather than CYP2C9.

Key words: cynomolgus monkey, CYP2C43, heterologous expression, liver microsomes, reconstitution.

Abbreviations: DLPC, dilauroylphosphatidylcholine; APMSF, 4-(2-aminoethyl)benzenesulfonyl fluoride HCl; CYP, cytochrome P450; IPTG, isopropyl-β-D-thiogalactopyranoside.

Cytochrome P450s (CYPs), present in the endoplasmic reticulum of mammalian liver cells, play an important role in the oxidative metabolism of numerous xenobiotics as well as endogenous compounds (*1*, *2*). In human liver, the CYP2C subfamily, which consists of CYP2C8, CYP2C9, CYP2C18, and CYP2C19 (*3*), accounts for roughly 20% of total CYPs (*4*) and is therefore of considerable research interets. Cynomolgus and rhesus monkeys have been widely used as experimental primates in preclinical drug studies, yet our understanding of the monkey CYP2C subfamily is limited. CYP2C isoforms have been purified from monkey liver (*5*–*7*), and two CYP2C isoforms have been cloned (CYP2C20 and CYP2C43 from cynomolgus and rhesus monkey liver, respectively) (*8*, *9*). CYP2C43 showed high amino acid sequence identity to CYP2C9 and CYP2C19. Monkey CYP2C43 cDNA was expressed in yeast and exhibited (*S*)-mephenytoin 4′-hydroxylation activity, a marker for human CYP2C19 (*9*). In this study, we cloned CYP2C43 cDNA from cynomolgus monkey liver and expressed it in *Escherichia coli*. The expressed protein was purified, and its substrate specificity was examined using CYP2C9 and CYP2C19 marker substrates.

MATERIALS AND METHODS

Apparatus—The HPLC system consisted of Hitachi instruments (HITACHI, Tokyo, Japan): an AS-4000 autosampler with a 100 μl injector loop, an L-6200 intelligent pump, and an L-4200 UV-VIS detector of variable wavelengths. The system was controlled through a D-6000 HPLC interface module.

Chemicals—Progesterone, testosterone, and androstenedione were purchased from Wako Pure Chemicals (Osaka, Japan). Deoxycorticosterone was obtained from Sigma (St. Louis, MN). Diclofenac sodium salt, 4′-hydroxydiclofenac, (*S*)-mephenytoin, 4′-hydroxymephenytoin, hydroxytolbutamide, and tolbutamide were purchased from Ultrafine Chemicals (Manchester, UK). Liver microsomes were obtained from Xeno Tech Inc. (Kansas City, KS). Cryopreserved primary hepatocytes of male cynomolgus monkeys were obtained from In Vitro Technologies (Baltimore, MD). Cytochrome b_5 was purchased from Pan Vera Corp. (Madison, WI). The pCW vector was a gift from Professor F. W. Dahlquist (University of Oregon, Eugene). Other chemicals were of the highest purity available.

Cloning of CYP2C43 cDNA—Total RNA was extracted from the cryopreserved male cynomolgus monkey hepatocytes using Isogen (Nippon Gene, Toyama, Japan), and the RNA was reverse-transcribed to yield cDNAs using an RNA PCR Kit (AMV) Ver.2.1 (TAKARABIO INC., Shiga, Japan). The resultant cDNAs were then amplified with KOD polymerase (TAKARABIO INC., Shiga, Japan). The sequences of the oligonucleotides used for PCR, 5′-ATGGATTCTCTTGTGGTCCT-3′ and 5′-AAAGGTGGATAACGCCCTGG-3′, were designed based on the cDNA sequence of CYP2C43 reported by Matsunaga *et al*. (*9*). The PCR products were then cloned into pCR4-TOPO

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(In Vitrogen, San Diego, CA), and the full-length DNA sequence was determined using a 5500-L DNA Sequencer (HITACHI, Tokyo, Japan).

Construction of a CYP2C43-Expressing Plasmid—The cloned CYP2C43 cDNA was modified by a PCR procedure. The N-terminal sequence was modified to achieve high-level expression in *E. coli*, and appropriate restriction sites were added for insertion into the expression vector. The sequence of the 5′-primer was 5′-CATATGGATTCTC-TTGTGGTGCTTGTG-3′ (to introduce an *Nde*I site prior to the start codon and to modify the N-terminal sequence), and the 3′-primer was 3′-GTCGACTTAGACAGGGAT-GAAGCACAGCAGGTA-5′ (to introduce a *Sal*I site down-stream of the stop codon). The modified cDNA was introduced into the pCW expression vector and then the vector was transformed into *E. coli* strain JM109.

Expression of CYP2C43 in E. coli and Preparation of a Membrane Fraction—*E. coli* JM109 transformed with the CYP2C43-expressing vector was grown overnight at 37°C in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin. An 80 ml aliquot was inoculated into 8 liters of Terrific Broth (TB) medium containing ampicillin (100 μg/ml) and 100 mM potassium phosphate buffer (pH 6.5) in a 10 liter fermenter, and then cultivated at 260 rpm with an aeration rate of 0.5 vvm at 30°C. Final concentrations of 1 mM δ-aminolevulinic acid and 1 mM isopropyl-β-D-thiogalactoside (IPTG) were added when the absorbance of the culture broth at the wavelength of 600 nm reached 0.2 and 0.3, respectively. The culture was continued for a further 24 h at 30°C. The cells were then harvested by centrifugation at 10,000 × *g* for 20 min. All subsequent steps were carried out at 4°C. The cells were resuspended in 100 mM Tris-HCl buffer (pH 7.4) containing 10 μM APMSF, 3 mg/liter leupeptin, 10 U/ml DNase I, and 0.3 mg/ml lysozyme. The cell suspension was gently stirred for 1 h and then centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended (*ca*. 0.5 g/ml) in 10 mM Tris-HCl (pH 7.6) containing 10 μM APMSF, 3 μg/ml leupeptin, 14 mM magnesium acetate, 20% (v/v) glycerol, and 60 mM potassium acetate. Cells were disrupted with an Astrason Ultrasonic Processor XL (Heat Systems, Farmingdale, NY), and the lysate was centrifuged at 10,000 × *g* for 20 min. The supernatant was then further centrifuged at 100,000 × *g* for 1 h. The pellet (membrane fraction) was resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 20% (v/v) glycerol, 10 μM APMSF, and 3 μg/ml leupeptin, and then stored at –80°C until use.

Detection of CYP2C43 Expression in E. coli—Expression of CYP2C43 was determined from the Fe^{2+}-CO vs Fe^{2+} difference spectrum according to the method described by Omura and Sato (*10*). Expression of CYP2C43 was also confirmed by Western blotting. The proteins were separated by SDS–polyacrylamide gel electrophoresis on a 7.5–15% gradient polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane in a wet-blotting apparatus. The membrane was blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan), and subsequently incubated with anti-rat CYP2C11 antibodies (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan) and then alkaline phosphatase-conjugated anti--rabbit goat-polyclonal antibodies. The membrane was incubated with 5-bromo-4-chloro-3-indolylphosphate and nitrotetrazolium blue, and then the visualized bands were analyzed (Fluor-S Multilmager, Bio-Rad). The protein concentrations were determined by the bicinchonic acid (BCA) procedure (Pierce Chemical Co., Rockford, IL).

Purification of Cecombinant CYP2C43—The *E. coli* membrane fraction was diluted to a protein concentration of 2.0 mg/ml in 20 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 1.0 mM DTT, 1.0 mM EDTA, and 1% (w/v) sodium cholate, and then stirred gently for 1 h. The solubilized membrane was centrifuged at 100,000 × *g* for 60 min at 4°C. A final concentration of 0.1 M NaCl was added to the supernatant, followed by loading onto a 2.5 cm × 20 cm Octyl-Sepharose CL-4B column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 100 mM potassium phosphate buffer (pH 7.25) containing 0.5% (w/v) sodium cholate and 0.5 M NaCl. The column was washed with 100 mM potassium phosphate buffer (pH 7.25) containing 0.5% (w/v) sodium cholate and 0.5 mM dithiothreitol (DTT). CYP2C43 was eluted with 100 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 1.5% (w/v) Triton N-101, and 0.5 mM DTT. The eluted fraction was pooled and diluted to a final concentration of 50 mM phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, and 0.5 mM DTT. The diluted fraction was applied to a 2.5 cm × 15 cm column of QAE-TOYOPEARL 550C (TOSOH Corp., Tokyo, Japan) that had been equilibrated with 20 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, and 1% (w/v) Triton N-101. CYP2C43 was eluted with a linear gradient of 0–1.5 M NaCl in 20 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, and 1% (w/v) Triton N-101. The eluted fractions were pooled and diluted to reduce the concentration of phosphate buffer to 5 mM. The diluted fraction was applied to a 2.5 cm × 2.0 cm hydroxyapatite column (Type I 400 μm; Bio-Rad Laboratories, Inc., Hercules, CA) that had been equilibrated with 5 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, and 1% (w/v) Triton N-101. CYP2C43 was eluted with a linear gradient of 5–750 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, and 1% (w/v) Triton N-101. The eluted fractions were pooled and dialyzed against 5 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol and 0.5% (w/v) sodium cholate. The dialyzed fraction was applied to a 2.5 cm × 2.0 cm column of hydroxyapatite that had been equilibrated with 5 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol and 0.5% (w/v) sodium cholate. The column was washed extensively with the same equilibration buffer, and then CYP2C43 was eluted with a linear gradient of 5–500 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol and 0.5% (w/v) sodium cholate.

Purification of Other Enzymes—NADPH-cytochrome P450 reductase was purified from phenobarbital-pretreated SD rat liver microsomes as described by Yasukochi and Masters (*11*). One unit was defined as 1 μmol of cytochrome *c* reduced per min. The specific activity of purified NADPH-cytochrome P450 reductase was 30 units/mg protein. CYP2C9 and CYP2C19 were expressed in *E. coli* and purified according to the method described previously (*12*).

The specific contents of purified CYP2C9 and CYP2C19 were 6.2 and 6.0 nmol/mg protein, respectively.

Enzyme Assays—The typical reaction mixture consisted of 50 pmol P450, 38 μM DLPC, 0.25 μM NADPH-P450 reductase, 50 mM HEPES buffer (pH 7.4), 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM NADPH, and the substrate, in a final volume of 200 μl. After pre-incubation at 37°C for 5 min, the reaction was started by the addition of 20 μl of 10 mM NADPH, and continued for 20, 30 or 60 min depending on the substrate (see below), with gentle stirring at 37°C. The final protein concentration for the microsomal metabolic assays was 0.1 mg protein/ml. The substrate concentrations were as follows: (*S*)-mephenytoin, 200 μM; tolbutamide, 1.6 mM; diclofenac, 1 mM; testosterone, 100 μM; and progesterone, 100 μM.

(*S*)-Mephenytoin 4′-hydroxylation was determined by the procedure described by Romkes *et al*., with minor modifications (*3*). After incubation for 30 min at 37°C, a 200 μl aliquot of acetonitrile was added to stop the reaction, and then the reaction mix was centrifuged at 3,000 rpm for 10 min. A 50 μl sample of the supernatant was analyzed by HPLC. The HPLC conditions were as follows: column, Capcell pack C18 AG120 (4.6 × 250 mm, 5 mm) (Shiseido Fine Chemicals, Tokyo, Japan); mobile phase, acetonitrile:10 mM potassium phosphate buffer (pH 7.4) (26:74, v/v); flow rate, 1.0 ml/min; and UV detection at 224 nm.

Tolbutamide *p*-methylhydroxylation was determined by the procedure described by Richardson *et al*., with modifications (*12*). After incubation for 60 min, the reaction was terminated by the addition of 50 μl 4 N HCl and 3 ml ethyl acetate. Following extraction with ethyl acetate, the extract was dried under nitrogen gas and dissolved in 50% (v/v) aqueous methanol. A 50 μl sample of the dissolved residue was analyzed by HPLC. The HPLC conditions were as follows: column, ODS-2 (4.6 × 150 mm, 5 μm) (GL Science Inc., Tokyo, Japan); mobile phase, acetonitrile:water:perchloric acid (310:690:1; v/v/v/); flow rate, 1.0 ml/min; and UV detection at 230 nm.

Diclofenac 4′-hydroxylation was determined by the procedure described by Tang *et al*. (*13*). The reaction was continued for 60 min at 37°C, and terminated by the addition of 0.5 ml of methanol and centrifugation at 3,500 rpm for 10 min. A 100 μl aliquot of the supernatant was analyzed by HPLC. The HPLC conditions were as follows: column, Inertsil ODS-3 (150 × 4.6 mm, 5μm) (GL Science Inc., Tokyo, Japan); mobile phase, acetonitrile:water:acetic acid (100:900:1; v/v/v/) (solvent A) and acetonitrile:water:acetic acid (900:100:1; v/v/v) (solvent B); flow rate, 1.0 ml/min; and UV detection at 280 nm. The initial mobile phase consisted of 40% solvent B, which was increased linearly to 90% from 5 min to 20 min.

Testosterone 17-oxidation and progesterone 21-hydroxylation were determined by the procedures described by Yamazaki and Shimada (*14*). The reaction was terminated after 20 min incubation at 37°C by the addition of 1ml of ethyl acetate, and a 100 μl aliquot of 20 μM 17α-methyltestosterone in methanol was added as an internal standard. Following extraction with ethyl acetate, the extract was dried under nitrogen gas. The dried residue was reconstituted in 25% (v/v) aqueous methanol, and then a 100 μl sample of the dissolved residue was assayed by HPLC. The HPLC conditions were as follows: column, TSK Gel Super ODS (4.6 × 100 mm, 5 μm) column (TOSOH Corporation, Tokyo, Japan); mobile phase, acetonitrile:water:methanol (2:58:40; v/v/v/) (solvent A) and acetonitrile:water:methanol (32:28:40; v/v/v) (solvent B); flow rate, 1.0 ml/min; and UV detection at 245 nm. The ratio of solvent B in the mobile phase was increased linearly from 0% to 27% over 5 min, maintained at 100% between 5 min to 10 min, decreased from 100% to 0% between 10 min to 11 min, and kept at 0% between 11 min to 16 min.

Mass Spectrometry and Data Acquisition—Metabolites of testosterone and progesterone were analyzed by LC-MS-MS using a ThermoFinnigan LCQ ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization source. HPLC was carried out on an ODS-3 column (2.1 × 150 mm, 5 μm) under the same conditions as described for the enzyme assays except that the flow rate was reduced to 0.2 ml/min. The instrument parameters were individually optimized to maximize the transition of the precursor ions to the most abundant product ion for each metabolite, and the dwell time was set at 400 ms for each transition. Analysis of the tandem MS experiment data was performed with ThermoFinnigan Xcalibur version 1.1 software.

Statistical Analysis—Kinetic parameters (K_m and V_{max}) of the reactions catalyzed by purified CYP2C isoforms were analyzed using KaleidaGraph (Synergy Software, Reading, PA), which was designed for non-linear regression analysis.

RESULTS

Monkey CYP2C43 cDNA—A CYP2C43 cDNA clone containing a complete open reading frame was isolated from primary hepatocytes of a male cynomolgus monkey. Compared with the sequence reported by Matsunaga *et al*. for rhesus monkeys (*9*), there were four amino acid differences; C334A (Leu112Ile), G1031A (Arg344His), T1263A (Asn421Lys), and A1444C (Ile482Val). However, Lle112, His344 and Lys421 were also found in CYP2C9 and CYP2C19 (Fig. 1 and Table 1). The homologies of the nucleotide and deduced amino acid sequences of CYP2C43 were compared with those of human CYP2C9 and CYP2C19 (Fig. 1 and Table 2). CYP2C43 is highly homologous to human CYP2C9 and CYP2C19, with 95.2% and 93.9% nucleotide sequence identity and 92.8% and 90.6% amino acid identity, respectively.

Purification of CYP2C43 Expressed in E. coli—The expression level of CYP2C43 exceeded 1,000 nmol P450 per liter in culture medium (data not shown). Table 3 summarizes the procedure used to purify CYP2C43 from the cDNA-expressing *E. coli* membrane fraction. The purified CYP2C43 appeared as a single major band on an SDS–polyacrylamide gel, the apparent molecular weight being 50 kDa (Fig. 2A). The specific content of purified CYP2C43 was 11.5 nmol P450/mg protein, and the total recovery from the solubilized membrane was 4.9%. The CO-reduced difference spectrum of the purified CYP2C43 showed a characteristic absorption peak at 450 nm (Fig. 3). Anti-CYP2C11 antibodies cross-reacted with both human CYP2C isoforms and CYP2C43. Slight differences were observed in the apparent molecular weights of CYP2C9, CYP2C19 and CYP2C43 (Fig. 2B), as noted for the highly homologous CYP2C isoforms (*7*, *15*).

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          10        20        30        40        50        60        70
2C43 MDSLVVLVLCLSCLLLLSLWRQRSGRGKLPPGPTPLPVIGNILKIGIKDVSKSLTNLSKVYGPVFTLYFG
2C19   PF              I S                 Q D           I
2C9                      S                 Q    I

          80        90        100       110       120       130       140
2C43 LERMVVLHGYEAVKEALIDLGEEFSGRGHFPLFERANRRFGIVFSNGKRWKEIRRFSLMTLRNFGMGKRS
2C19           V                   A    G
2C9    KPI                       I  A    G        K

         150       160       170       180       190       200       210
2C43 IEDRVQEEARCLVEELRKTKASPCDPTFILGCAPCNVICSIIFHKRFDYKDQQFLNLMEKFNENAKILSS
2C19                                     Q                 L   IR V T
2C9                                                       L   I

         220       230       240       250       260       270       280
2C43 PWIQIYNNFSPIIDYFPGTHNKLLKNIAFVKSYILEKVKEHQESMDMNNPRDFIDCFLIKMEKEKHNQQS
2C19      C  PT             L  ME D          I                    Q
2C9       C                  V  M                   Q       M        P

         290       300       310       320       330       340       350
2C43 EFNIENLENTAVDLFAAGTETTSTTLRYALLLLLKHPEVAAKVQEEIEHVIGRNRSPCMQDRSHMPYTDA
2C19   T   VI  A  LG                    T       R             G
2C9    T  S        G                    T       R

         380       370       380       390       400       410       420
2C43 VVHEIQRYIDLLPTSVPHAVTCDVKFRNYLIPKGTTILISLTSVLRDNKEFPNPEMFDPRHFLDEGGNFK
2C19     V      I  L                    T     H
2C9      V         L      I                    H           H

         430       440       450       460       470       480       490
2C43 KSNYFMPFSAGKRICVGEALARMELFLFLTSILQNFNLKSLVDLKDLDTTPVFNGFVSVPPVYQLCFIPV*
2C19                  G          F          I P       V  A    F         *
2C9    K                 G                    P N     V  A    F         *
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Fig. 1. **Amino acid sequence alignment of monkey CYP2C43, human CYP2C19 and human CYP2C19.** Residues differing from CYP2C43 are indicated below the CYP2C43 sequence. An asterisk indicates the termination codon.

Table 1. **Substitution of amino acids in CYP2C43 cloned in this study.**

	Amino acid residue			
	112	344	421	482
CYP2C43 (*9*)	L	R	N	I
CYP2C43 (this study)	I	H	K	V
CYP2C9	I	H	K	F
CYP2C19	I	H	K	F

Amino acid positions and substituted amino acids are shown.

Table 2. **Percentage identity for nucleotide and amino acid sequences of human CYP2C9, human CYP2C19, and cynomolgus CYP2C43.**

		CYP2C19	CYP2C43
CYP2C9	Nucleotide	94.8%	95.2%
	Amino acid	91.4%	92.8%
CYP2C19	Nucleotide		93.9%
	Amino acid		90.6%

Metabolic Properties of CYP2C9, CYP2C19 and CYP2C43 as to Human CYP2C9 and CYP2C19 Marker Substrates—Diclofenac, (*S*)-mephenytoin, and tolbutamide were used as isoform-specific marker substrates for human CYP2C. Diclofenac indicates CYP2C9-dependent 4′-hydroxylation, (*S*)-mephenytoin indicates CYP2C19-dependent 4′-hydroxylation, and tolbutamide indicates CYP2C9- and CYP2C19-dependent *p*-methylhydroxylation. As shown in Table 4, CYP2C9 exhibited high activity for tolbutamide *p*-methylhydroxylation (6.5 nmol/min/nmol P450) and diclofenac 4′-hydroxylation (9.22 nmol/min/nmol P450), while CYP2C19 showed high activity for (*S*)-mephenytoin 4′-hydroxylation (1.43 nmol/min/nmol P450). CYP2C9 showed reduced activity for tolbutamide *p*-methylhydroxylation (1.9 nmol/min/nmol P450) compared with CYP2C19 (6.5 nmol/min/nmol P450). On the other hand, CYP2C43 showed reduced activity for diclofenac 4′-hydroxylation (0.81 nmol/min/nmol P450) and (*S*)-mephenytoin 4′-hydroxylation (0.06 nmol/min/nmol P450). Tolbutamide metabolic activity was undetectable with CYP2C43.

Table 3. **Purification of CYP2C43 from cDNA-expressing *E. coli* membrane fraction.**

Purification step	Protein (mg)[a]	CYP (nmol)	Specific content (nmol/mg protein)[a]	Yield (%)
Membrane	5,910	1,770	0.30	100
Solubilized membrane	1,620	566	0.35	32
Octyl-Sepharose	84.4	170	2.01	9.6
QAE-Sepharose	42.1	127	3.01	7.2
Hydroxyapatite	7.50	85.9	11.5	4.9

[a]These values should be considered nominal.

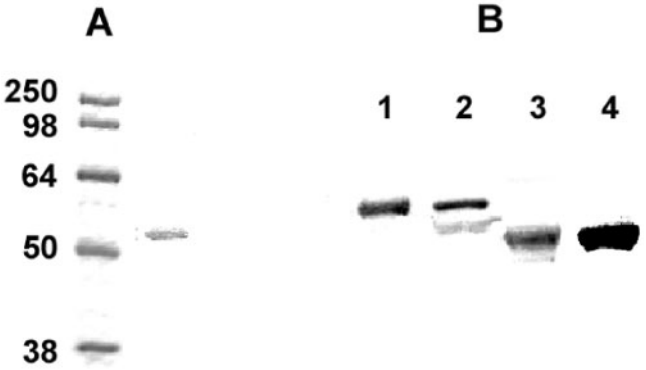

Fig. 2. **Purity and immunoreactivity of purified CYP2C43 from the cDNA-expressing *E. coli* membrane fraction.** (A) SDS–Polyacrylamide gel electrophoresis of purified CYP2C43. 0.5 μg of purified CYP2C43 was run on a 5–12.5% gel. Left lane, marker proteins; right lane, purified CYP2C43. Each lane contained 150 pmol of cytochrome P450. (B) Immunoblotting was performed for purified CYP2C9 (lane 1), purified CYP2C19 (lane 2), purified CYP2C43 (lane 3), and CYP2C11-expressing baculovirus microsomes (lane 4, control), using anti-CYP2C11 antibodies. After SDS–polyacrylamide gel electrophoresis, proteins were transferred to a PDVF membrane. Alkaline phosphatase–conjugated anti-rabbit goat-polyclonal antibodies were used as secondary antibodies.

The same human CYP2C isoform–specific marker substrates were used to examine the metabolism in human, monkey, rabbit, guinea pig, hamster, rat, and mouse liver microsomes. The (*S*)-mephenytoin 4′-hydroxylation activity in human and monkey liver microsomes amounted to 44 and 61 pmol/min/mg protein, respectively, but was considerably lower in other animal species (Table 4).

Testosterone and Progesterone Metabolism with Purified CYP2C9, CYP2C19, and CYP2C43—LC-MS and HPLC were used to identify and quantify the metabolites of testosterone and progesterone after incubation with purified CYP2C isoforms and human liver microsomes. The metabolites were confirmed by mass spectrometry in comparing with authentic samples (Fig. 4). Testosterone and progesterone were metabolized to androstenedione and 21-hydroxyprogesterone, respectively, by both CYP2C43 and CYP2C19. In contrast, testosterone and progesterone were not metabolized by CYP2C9 (Fig. 5, A and B).

Kinetics of Steroid Hormone Metabolism with Purified CYP2C19 and CYP2C43—The typical kinetic patterns are presented for the metabolism of testosterone to androstenedione (Fig. 6) and progesterone to 21-hydroxy progesterone (Fig. 7), in a reconstitution system with CYP2C43 or CYP2C19. Simple Michaelis-Menten kinetic analysis was performed to estimate the affinity constant (K_m) and the maximum enzyme velocity (V_{max}) (Table 5). The apparent K_m and V_{max} values for testosterone 17-oxidation were 90 μM and 4.2 nmol/min/nmol P450 for CYP2C19, and 45 μM and 4.2 nmol/min/nmol P450 for CYP2C43. Similarly, the K_m and V_{max} values for progesterone 21-hydroxylation were 6.0 μM and 3.8 nmol/min/nmol P450 for CYP2C19, and 16 μM and 3.4 nmol/min/nmol CYP for CYP2C43.

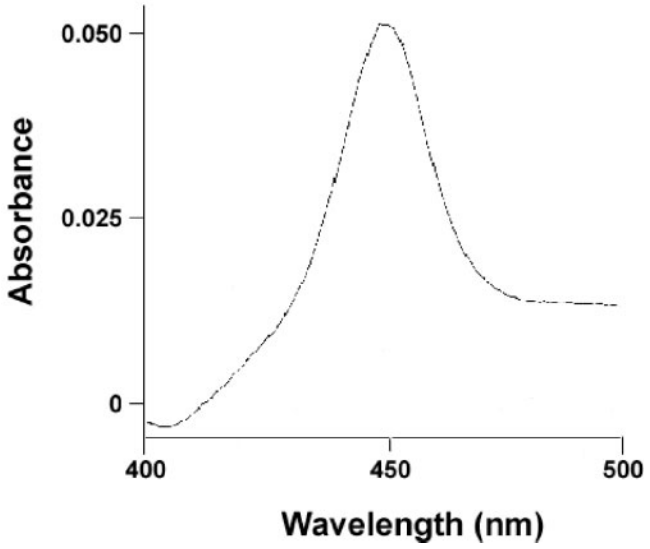

Fig. 3. **CO-difference spectrum of purified CYP2C43 from cDNA-expressing *E. coli* membrane fraction.** Purified CYP2C43 was diluted 10-fold in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol. The CO-difference spectrum was obtained by the method of Omura and Sato (*10*).

DISCUSSION

The deduced amino acid sequence of CYP2C43 cDNA cloned from cynomolgus monkey in this study showed 92.8% and 90.6% identity to human CYP2C9 and CYP2C19, respectively (Table 2 and Fig. 1). Four amino acid substitutions were found compared with the previously published sequence for rhesus monkey (*9*). Amino acid residues Leu112, Arg344, Asn421 and Ile482 in the rhesus sequence were substituted by Ile, His, Lys, and Val, respectively (Table 1). Residues 112, 344, and 421 in this study were identical those in human CYP2C9 and CYP2C19 (Table 1), and human CYP2C8 and CYP2C18 (*16*). Residue 482 differed between the CYP2C isoforms: Val in this study, Ile in rhesus monkey [Matsunaga *et al*. (*9*)], Phe in human CYP2C9 and CYP 2C19 (Table 1), Ser in human CYP2C8, and Leu in human CYP2C18 (*16*). We cannot at present explain the amino acid substitutions in the two CYP2C43 sequences cloned from cynomolgus and rhesus monkey. Inter-individual and inter-strain differences are two obvious possibilities. However, repeated sequencing of different colonies containing cDNA produced by PCR using nucleotides designed to amplify CYP2C43 showed an identical cDNA sequence. Furthermore, the cloned CYP2C43 contained Ile112, His344, and Lys421, which were the same as in human CYP2C8, 9, and 19, and especially Lys421 has been reported to be conserved in the CYP2C subfamily (*17–20*). Therefore, we conclude

Table 4. **Catalytic activities of recombinant CYP2C9, CYP2C19, and CYP2C43 expressed in *E. coli* and in liver microsomes.**

Enzyme	Diclofenac 4′-hydroxylation	(*S*)-Mephenytoin 4′-hydroxylation	Tolbutamide *p*-methylhydroxylation
Recombinant CYP isoforms			
		(nmol/min/nmol P450)	
CYP2C9	9.22	ND[a]	6.5
CYP2C19	0.42	1.43	1.9
CYP2C43	0.81	0.06	ND[a]
Liver microsomes			
		(pmol/min/mg protein)	
Human	0.92	44	1.59
Monkey	0.35	61	1.12
Dog	0.10	13	ND[b]
Rabbit	0.33	4.3	2.17
Guinea pig	0.22	2.4	0.19
Hamster	0.75	2.1	0.50
Rat	0.13	5.8	1.23
Mouse	0.17	10	0.20

Results shown are the means for duplicate experiments. ND: not detected. [a]Detection limit: 10 pmol/min/nmol P450. [b]Detection limit: 0.01 pmol/min/mg protein.

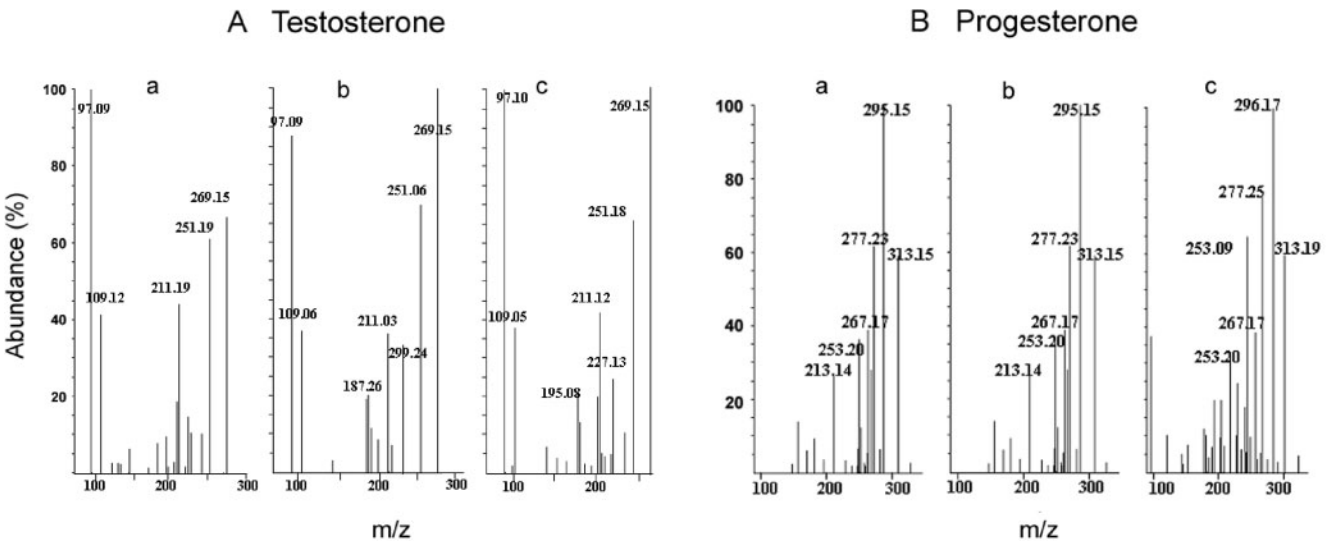

Fig. 4. **Product ion mass spectra of the MH^+ obtained from the metabolite after incubation of purified CYP2C9 and CYP2C43 with testosterone (A) and progesterone (B) on reconstitution, and authentic samples.** (A) a, androstenedione; b, CYP2C19 metabolite; c, CYP2C43 metabolite. (B) a, 21-hydroxyprogesterone; b, CYP2C19 metabolite; c, CYP2C43 metabolite.

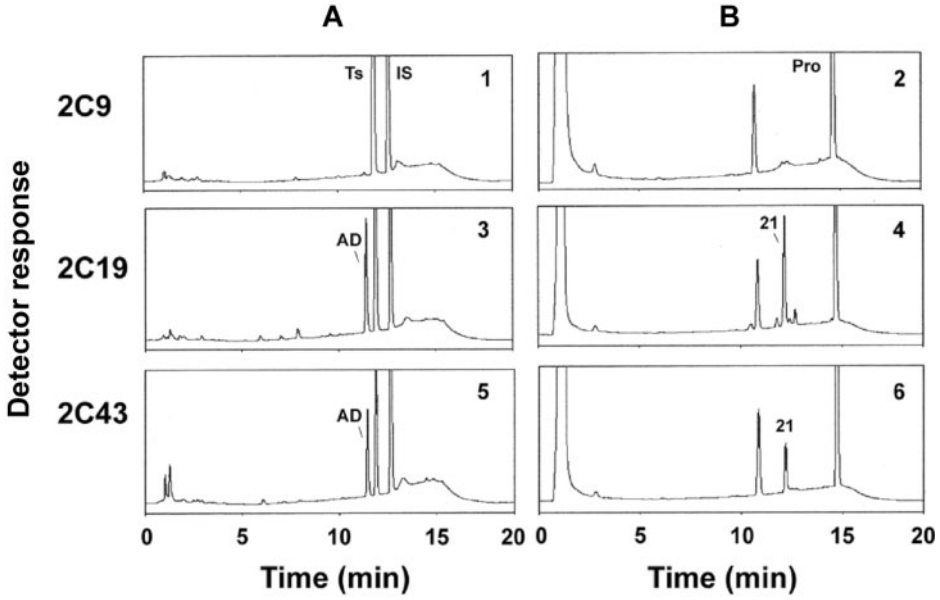

Fig. 5. **Representative HPLC chromatograms of metabolites formed after incubation of purified CYP2C9, CYP2C19, and CYP2C43 with testosterone and progesterone.** (A) 50 μM testosterone was incubated with 50 pmol of purified CYP2C9, CYP2C19, or CYP2C43 at 37°C for 30 min. 1, CYP2C9; 3, CYP2C19; 5, CYP2C43; AD, androstenedione; Ts, testosterone; IS, 17α-methyltestosterone (internal standard). (B) 50 μM progesterone was incubated with 50 pmol of purified CYP2C9, CYP2C19, or CYP2C43 at 37°C for 30 min. 2, CYP2C9; 4, CYP2C19; 6, CYP2C43. Pro, progesterone; 21, 21-hydroxyprogesterone.

that the amino acid sequence of CYP2C43 cDNA cloned in this study represents that of the male cynomolgus monkey.

We used marker substrates for human CYP2C9 and CYP2C19 to compare the metabolic activities with those of cynomolgus CYP2C43 (Table 4). (*S*)-Mephenytoin 4′-hydroxylation, testosterone 17-oxidation, and progesterone 21-hydroxylation were all catalyzed by human CYP2C19. Diclofenac 4′-hydroxylation was mediated by human CYP2C9, while tolbutamide *p*-methylhydroxylation was catalyzed by human CYP2C9 and CYP2C19. Cynomolgus CYP2C43 exhibited minimal (*S*)-mephenytoin 4′-hydroxylase activity, *i.e.*, 4.2% that of human CYP2C19. Testosterone and progesterone were metabolized (to androstenedione and 21-hydroxyl progesterone, respectively) by CYP2C43 and CYP2C19 with similar V_{max} values (Table 5). CYP2C43 showed low diclofenac 4′-hydroxylase activity, *i.e.*, 8.8% that of human CYP2C9. However, tolbutamide was not metabolized by CYP2C43. The results show that the substrate specificity and metabolic activity of CYP2C43 resemble those of human CYP2C19 in

(*S*)-mephenytoin 4′-hydroxylation, diclofenac 4′-hydroxylation, testosterone 17-oxidation and progesterone 21-hydroxylation. Thus, CYP2C43 can be considered to be similar to human CYP2C19 rather than to CYP2C9, in terms of human CYP2C marker substrate metabolism.

As previously reported (*14*, *21*), human and monkey liver microsomes exhibited much higher activity for (*S*)-mephenytoin 4′-hydroxylation than microsomes from other animal species (including dog, rabbit, guinea pig, hamster, rat and mouse). Notably, monkey liver microsomes were 1.8-fold more active for (*S*)-mephenytoin 4′-hydroxylation than those of humans. Yamazaki and Shimada showed that testosterone 17-oxidation activity in human and monkey liver microsomes was inhibited by anti-CYP2C9 antibodies. These antibodies were inhibitory to both CYP2C9 and CYP2C19 catalytic activities (*14*), but as shown here, CYP2C9 lacks testosterone 17-oxidase activity. Therefore, CYP2C19 is likely to play a major role in testosterone 17-oxidation in human liver microsomes. The testosterone 17-oxidase activity in monkey liver microsomes was 6.7-fold higher than that in man (date not shown). Assuming that testosterone 17-oxidation in monkey liver microsomes is catalyzed by CYP2C43 (by analogy with CYP2C19 in human liver microsomes), the higher (*S*)-mephenytoin 4′-hydroxylase activity in monkey liver microsomes compared with in human microsomes might not be accounted for by CYP2C43 in monkey liver. The expression level of CYP2C43 in monkey liver is unknown. However, a discrepancy in (*S*)-mephenytoin 4′-hydroxylase activity, high in monkey liver microsomes but low in purified CYP2C43 compared with that in humans, could imply that another CYP possessing this activity is present in monkey liver. A useful paradigm may be the two CYP2D isoforms recently isolated from marmoset liver (*22*). Marmoset CYP2D19 and CYP2D30 showed 91.6% and 96.2% amino acid sequence identity with human CYP2D6, respectively. However, while debrisoquine 4-hydroxylation catalyzed by CYP2D30 was similar to that by human CYP2D6, CYP2D19 lacked this activity. For (*S*)-mephenytoin 4′-hydroxylation, it has not been confirmed whether similarly distinct CYP isoform(s) exist in cynomolgus monkey liver. Recently, the crystal structure of CYP2C9 was elucidated (*23*, *24*). Given the high amino acid sequence identity with CYP2C9, cynomolgus CYP2C43 may well assume a similar structure. Studies to identify the critical amino acids responsible for substrate specificity are currently under way in our laboratory.

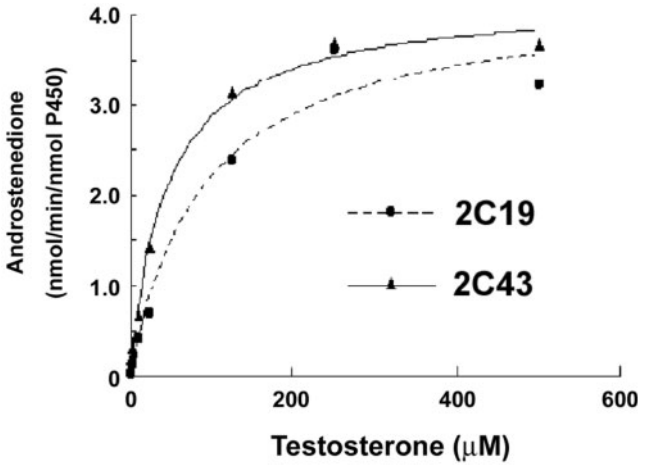

Fig. 6. **Kinetics of testosterone metabolism.** Testosterone was metabolized by CYP2C19 and CYP2C43 on reconstitution as described under "MATERIALS AND METHODS."

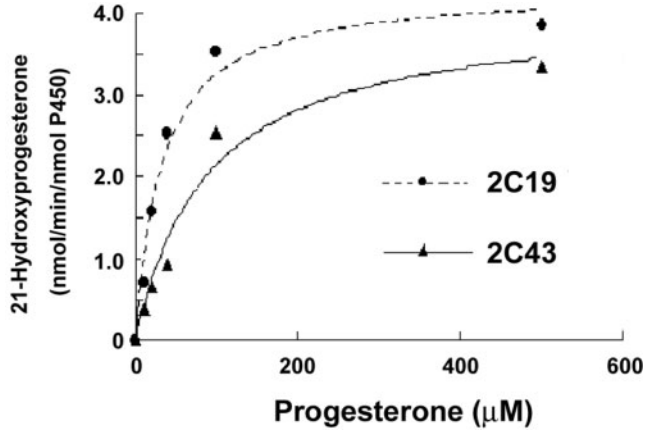

Fig. 7. **Kinetics of progesterone metabolism.** Progesterone was metabolized by CYP2C19 and CYP2C43 on reconstitution as described under "MATERIALS AND METHODS."

In conclusion, CYP2C43, which exhibits high amino acid sequence identity to human CYP2C9 and CYP2C19, was cloned from cynomolgus monkey liver and expressed in *E. coli*. The purified CYP2C43 oxidized testosterone and progesterone, to yield androstenedione and 21-hydroxylprogesterone, respectively, in a similar manner to CYP2C19. However, it showed no activity for tolbutamide *p*-methylhydroxylation and lower diclofenac 4′-hydroxylase activity. It exhibited low but significant (*S*)-mephenytoin 4′-hydroxylase activity, which is canonically attributed to CYP2C19 in man. Together these results indicate that the substrate specificity and enzyme activity of cynomolgus CYP2C43 are similar to those of human CYP2C19.

We would like to thank Dr. Norio Shimamoto for the helpful discussions. We are also grateful to Mr. Yutaka Kiyota and

Table 5. **Michaelis-Menten kinetic parameters of testosterone 17-oxidation and progesterone 21-hydroxylation by purified CYP2C19 and CYP2C43 from cDNA-expressing *E. coli* membranes.**

CYP isoforms	Testosterone 17-oxidation			Progesterone 21-hydroxylation		
	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m
CYP2C19	90	4.2	0.047	6.0	3.8	0.63
CYP2C43	45	4.2	0.093	16	3.4	0.22

V_{max} is expressed as nmol/min/nmol P450. K_m is expressed as μM. Substrate concentrations were varied from 10 μM to 500 μM.

Dr. Teruaki Okuda for the chemical analysis of metabolites, and Dr. Sumie Yoshitomi and Ms. Keiko Ikemoto for their technical guidance throughout this study. We also thank Dr. Kenji Okonogi and Dr. Tetsuo Miwa for their encouragement throughout this study, and express our gratitude to Dr. E. Dahlquist for providing the pCW expression vector.

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