Expression and purification of human cholesterol 7α-hydroxylase in *Escherichia coli*

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Abstract Cholesterol 7α-hydroxylase (P450c7) is the first and rate-limiting enzyme in bile acid biosynthesis and is the product of a cytochrome P450 gene, *CYP7*. We have previously reported the cloning of a full-length human cholesterol 7α-hydroxylase cDNA (Karam, W. G., and J. Y. L. Chiang. 1992. *Biochem. Biophys. Res. Commun.* **185:** 588–595). Using this clone in a polymerase chain reaction, we have generated a cDNA (H7α1.5) in which the codons for the N-terminal 24 amino acid residues were deleted. The translational product of this cDNA would be a truncated protein, P450c7(Δ 2–24) with a hydrophilic NH_2-terminal sequence, Met-Ala-Arg-Arg-Arg-Gln... This cDNA was cloned into the expression vector pJL and the construct pJL/H7α1.5 was transformed into *E. coli* strain TOPP3. We have also ligated a truncated rat cholesterol 7α-hydroxylase cDNA obtained previously (Li, Y. C., and J. Y. L. Chiang. 1991. *J. Biol. Chem.* **266:** 19186–19191) into the pJL vector and have transformed this construct (pJL/R7α1.5) into *E. coli* strain MV1304. Both of these systems expressed functional cholesterol 7α-hydroxylase in *E. coli*. A fivefold improvement in the expression of rat enzyme over the previous expression system was obtained. About 70–80% of the truncated human P450 in the clear lysate was localized in the cytosol. The truncated human and rat P450c7(Δ 2–24) were purified to homogeneity. Reconstitution of cholesterol 7α-hydroxylase activity using purified rat or human P450c7(Δ 2–24) showed a similar K_m of 6 and 7 μM for cholesterol, a V_{max} of 0.13 and 0.14 nmol/min, and a turnover number of 1.3 and 1.5 per min, respectively. Immunoblotting experiment revealed that a polyclonal antibody raised against rat microsomal cholesterol 7α-hydroxylase recognized both rat and human P450c7(Δ 2–24). This expression system provides a method for isolation of a large quantity of purified and catalytically active cholesterol 7α-hydroxylase for the study of structure and function of this important enzyme in bile acid synthesis and cholesterol homeostasis.—**Karam, W. G., and J. Y. L. Chiang.** Expression and purification of human cholesterol 7α-hydroxylase in *Escherichia coli*. *J. Lipid Res.* 1994. **35:** 1222–1231.

Supplementary key words bile acid synthesis • cytochrome P450 • cDNA

Cholesterol 7α-hydroxylase (P450c7) (EC.1.14.13.17), the product of the *CYP7* gene (1), is the first and rate-limiting enzyme in the conversion of cholesterol to bile acids in the liver (2, 3). This enzyme activity is feedback-regulated by hydrophobic bile acids returning to the liver via the enterohepatic circulation of the bile (2) and is stimulated by treatment with cholestyramine, a bile acid sequestrant, and by feeding a high cholesterol diet to rats (4). Recent breakthroughs in the purification of rat cholesterol 7α-hydroxylase (5, 6) and cloning of cDNAs (4, 7, 8) and the gene encoding rat cholesterol 7α-hydroxylase (9, 10) have contributed to the understanding of molecular mechanism of regulation of bile acid synthesis by this rate-limiting enzyme. It has been demonstrated that the changes in microsomal cholesterol 7α-hydroxylase activity and enzyme levels parallel the steady-state mRNA levels and the rate of gene transcription in rat livers (4, 5, 7, 8, 11). However, regulation of cholesterol 7α-hydroxylase in the human liver has not been studied as extensively as in the rat liver. This is partly due to the lack of suitable human liver tissues and the expression of an extremely low level of cholesterol 7α-hydroxylase activity in human liver, which prevented the purification and characterization of human cholesterol 7α-hydroxylase. Two laboratories have reported partial purification of the human cholesterol 7α-hydroxylase from human liver; however, kinetic characteristics of the human enzyme have not been studied (12, 13). Full-length cDNAs encoding human cholesterol 7α-hydroxylase have been cloned recently by screening human liver cDNA libraries using rat cDNAs as hybridization probes (14, 15). Recent advances in the expression of membrane-bound proteins such as P450 isozymes in heterologous system, *E. coli*, have provided a unique method for the production and isolation of human enzymes (16–18).

Abbreviations: PCR, polymerase chain reaction; LB broth, Luria Bertani broth; IPTG, isopropyl-β-D-thiogalactopyranoside; HPLC, high performance liquid chromatography; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethyl sulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; δ-ALA, delta-aminolevulinic acid; OAS-4B, octylamino-Sepharose B; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammoniodimethylammonio]-1-propanesulfonate.

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In this communication, we report the development of an *E. coli* expression system for a human cholesterol 7α-hydroxylase based on our previous strategy of expressing a truncated, soluble, and catalytically active rat cholesterol 7α-hydroxylase in *E. coli* (16). The bacteria-expressed human cholesterol 7α-hydroxylase was purified and its kinetic and immunochemical properties were characterized.

EXPERIMENTAL PROCEDURES

Materials

All bacterial culture media were purchased from Difco (Dearborn, MI); restriction enzymes were from Bethesda Research Laboratories (Gaithersburg, MI or Promega (Madison, WI); expression vector pKK233-2 was from Pharmacia (Piscataway, NJ). The pJL expression vector and *E. coli* strain MV1304 were a generous gift from Drs. M. J. Coon and J. R. Larson of the University of Michigan, Ann Arbor (17). *E. coli* strains TOPPs and XLI-blue and pBluescript SK+ II plasmid were purchased from Stratagene (La Jolla, CA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Fisher (Pittsburgh, PA); δ-aminolevulinic acid (δ-ALA), cholesterol, 20α-hydroxycholesterol, and L-dilauroylglyceryl-3-phosphorylcholine were from Sigma (St. Louis, MO); cholesterol oxidase (*Streptomyces*) was from Calbiochem (San Diego, CA). Octylamino-Sepharose 4B (OAS-4B) gel was prepared as described previously (19). NADPH-cytochrome P450 reductase was purified from rabbit liver microsomes as described previously (20). Hydroxylapatite was obtained from Bio-Rad (Richmond, CA); reagents for polymerase chain reaction (PCR) were from Perkin-Elmer Cetus (Norwalk, CT); Immobilon-P membranes were from Millipore (Bedford, MA). Sequenase version 2 sequencing kit was obtained from the United States Biochemicals Corp. (Cleveland, OH). Molecusol (45% aqueous solution of 2-hydroxypropyl-β-cyclodextrin) was purchased from Pharmatech Inc. (Alachua, FL). Gene-Clean kit was from BIO101 (La Jolla, CA). All other chemicals used were of the highest quality available commercially.

Construction of the human cholesterol 7α-hydroxylase expression system

Cloning and sequencing of the full length human cholesterol 7α-hydroxylase cDNA (pHC7F) in our laboratory has been reported previously (15). The 3'-primer used was 5'-GCCATGGCCGTAATATCATCTAG-3', complementary to nucleotides 1599 to 1612 of the 3'-flanking region of pHC7F. The 5'-primer was 5'-GCCATGGCCAGGAGA AGGCAAACGGGT-3', corresponding to nucleotides 132 to 149 of pHC7F. Both PCR primers have a *NcoI* restriction site, "GCCATG," which contains the ATG start codon. PCR was done using these two primers which were annealed to a template pHC7F to synthesize a modified cDNA (H7α1.5) lacking the first 24 amino acid codons of the N-terminal hydrophobic domain of human cholesterol 7α-hydroxylase. The concentration of each primer was 1 μM and 2 ng of linearized pHC7F was used per reaction in a final volume of 100 μl. A Perkin-Elmer Cetus DNA thermal cycler was used for PCR. Each thermal cycle included a denaturing step at 94°C for 1 min, an annealing step at 65°C for 1 min, an extension step at 72°C for 3 min, and an autoextension segment of 3 sec, for 30 cycles, and a final extension step of 10 min at 72°C. The PCR product was purified by a 1% agarose gel electrophoresis, followed by extraction with glass powder (Gene-Clean) and cut with *NcoI*. cDNA was ligated into pBluescript SK+ II plasmid linearized with *NcoI,* transformed into *E. coli* strain XLI-blue and sequenced to confirm the nucleotide sequences. The cesium chloride-purified plasmid was digested with *NcoI* and was then ligated into *NcoI*-digested expression vector pKK233-2 or the pJL expression plasmid. These constructs were then transformed into *E. coli* strains XLI-blue, MV1304, or a panel of TOPPs. **Figure 1A** shows the plasmid construct of the human P450c7(Δ 2-24) expression vector, pJL/H7α1.5.

Construction of the rat cholesterol 7α-hydroxylase expression system

The truncated rat 7α-hydroxylase cDNA developed by Li and Chiang (16) was cut out of the pKK233-2 expression plasmid with *NcoI* and religated into the *NcoI* site of the pJL expression plasmid. This construct was then transformed into *E. coli* strain MV1304. Figure 1B shows the plasmid construct of the rat P450c7(Δ 2-24) expression vector, pJL/R7α1.5.

Expression of human cholesterol 7α-hydroxylase P450c7(Δ 2-24) in *E. coli*

Eight liters of "terrific broth" (2.4% yeast extract, 1.2% tryptone, 0.4% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) containing 100 mg ampicillin/liter were inoculated with a 6-h pilot culture of TOPP3-pJL/H7α1.5 in Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 172 mM sodium chloride) containing the same concentration of ampicillin. The culture was grown at 37°C with shaking at 200 rpm, until the OD_{600} reached 0.4-0.6. IPTG and δ-ALA were added to a final concentration of 1 mM and 0.2 mM, respectively. Induction of protein synthesis was carried out at 30°C with shaking at 150 rpm for 15-18 h.

Expression of rat cholesterol 7α-hydroxylase P450c7(Δ 2-24) in *E. coli*

Four liters of "terrific broth" containing 100 mg ampicillin/liter were inoculated with a 6-h culture of MV1304-pJL/R7α1.5 in LB, containing the same concentration of ampicillin. The culture was grown at 37°C with

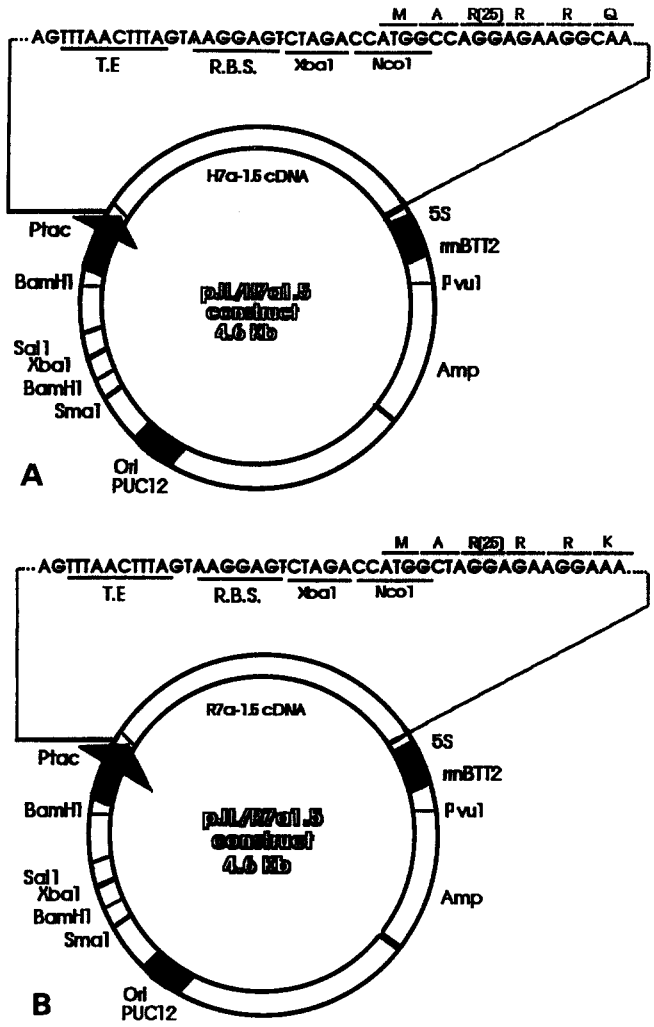

Fig. 1. Expression plasmid constructs of the human and rat P450c7(Δ 2–24) enzymes. The human and rat 1.5 kb cDNAs were ligated into the pJL vector at a unique *NcoI* site. R[25] refers to arginine codon number 25 in the full-length protein. The codons for the N-terminal hydrophobic amino acid segment preceding R[25] have been deleted. The ribosomal binding site [RBS] is preceded by a translation enhancer sequence [TE] derived from gene 10 of phage T7 (25). A: Human P450c7(Δ 2–24) expression vector construct, pJL/H7α1.5. B: Rat P450c7(Δ 2–24) expression vector construct, pJL/R7α1.5.

shaking at 200 rpm until the absorbance at 600 nm reached 0.6–0.8. IPTG was added to a final concentration of 1 mM and induction was carried out at 32°C with shaking at 200 rpm for 4 h.

Purification of the *E. coli* expressed human P450c7(Δ 2-24)

The purification procedure was based on the method developed previously (16) with some modification. Harvested cells were stored on ice for 20 min and then centrifuged at 4500 *g* for 10 min at 4°C. The pellet thus obtained was resuspended in 1/100th of the original volume in buffer A (100 mM potassium phosphate, pH 7.4, 0.5% sodium cholate, 20% glycerol, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), and 0.5 mM phenylmethyl sulfonylfluoride (PMSF)). The cells were then lysed in buffer A by adding 200 μg lysozyme/ml and occasionally triturating the resulting slurry for 1 h on ice. The supernatant fluid was collected after centrifugation of the total lysate at 100,000 *g* for 1 h at 4°C. The pellet was resuspended thoroughly in buffer A using 1/4th of the previously used volume and centrifuged again. One hundred units of deoxyribonuclease I were added to the combined supernatants and stored on ice overnight. All buffers used for chromatography contained 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.5 mM PMSF. The clear lysate was then applied to an Octylamino-Sepharose 4B column, (33 cm × 2.6 cm). The column was washed and eluted with buffer A. Assay of cholesterol 7α-hydroxylase activity in the column fractions was difficult because the inhibitory effect of sodium cholate which was present in the buffer, and because assay of 7α-hydroxylase activity in column fractions requires reconstitution with P450 reductase, which is very tedious. As no other P450 cytochrome is present in *E. coli,* column eluates were routinely monitored by absorbance at 416 nm which indicates the presence of P450c7 cytochrome. The pooled P450 fractions were diluted or dialyzed against buffer without sodium cholate and were assayed for 7α-hydroxylase activity by reconstitution assay as described. The P450 fractions containing 7α-hydroxylase activity were then dialyzed against 10 mM potassium phosphate buffer, pH 7.4, 0.2% sodium cholate, 0.2% Emulgen 911, and applied to a hydroxylapatite column (7.0 cm × 2.4 cm) equilibrated with the same buffer. The column was washed with 200 ml 10 mM potassium phosphate buffer, pH 7.4, 0.3% sodium cholate. This column was then washed with 150 ml 50 mM potassium phosphate buffer, pH 7.4, 0.3% sodium cholate and eluted with 300 ml 100 mM potassium phosphate buffer, pH 7.4, 0.3% sodium cholate. These fractions were combined, after which Emulgen 911 was added to a final concentration of 0.2%, and dialyzed against 10 mM potassium phosphate buffer, pH 7.4, 0.2% Emulgen 911. This enzyme preparation was then applied to a second hydroxylapatite column (3 cm × 0.5 cm) equilibrated with the same buffer and then eluted out with 360 mM potassium phosphate buffer, pH 7.4, 0.2% Emulgen 911. Enzyme preparations used subsequently in kinetic studies were dialyzed against 100 mM potassium phosphate buffer, pH 7.4, 0.2% Emulgen 911, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol.

Purification of the *E. coli* expressed rat P450c7(Δ 2-24)

Four liters of culture of MV1304-pJL/R7α1.5 were lysed as described above. The clear lysate was applied to an Octylamino-Sepharose 4B column (15 cm × 2.6 cm)

equilibrated with buffer A. The column was then washed and eluted with buffer A. The eluted fractions were dialyzed against 30 mM potassium phosphate, pH 7.4, 0.2% sodium cholate, 0.2% Emulgen 911, and applied to a hydroxylapatite column (5 cm × 2.4 cm) equilibrated with the same buffer. The column was then washed with 300 ml 50 mM potassium phosphate, pH 7.4, 0.2% sodium cholate, 0.2% Emulgen 911, and eluted with 180 mM potassium phosphate, pH 7.4, 0.2% sodium cholate, 0.2% Emulgen 911. Enzyme preparations used in kinetic studies were dialyzed against 100 mM potassium phosphate buffer, pH 7.4, 0.2% Emulgen 911, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol.

Subcellular distribution of human P450c7(Δ 2-24)

Two hundred ml TOPP3-pJL/H7α1.5 culture was grown and induced as described previously. The cells were lysed by lysozyme followed by sonication (20 sec pulse at 100 watts, on ice) in 5 ml 100 mM or 10 mM potassium phosphate buffer, pH 7.4, with or without 0.5% sodium cholate or 0.1% Triton X-100 (**Table 1**). The resulting lysate was centrifuged at 13,000 *g* at 4°C for 20 min. The supernatants obtained were centrifuged at 105,000 *g* for 1 h at 4°C. Both pellets were resuspended in respective buffer. P450s content in the pellets and supernatants were then determined by difference spectrum of the CO-adduct of the reduced P450.

Reconstitution of cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase activity of the purified enzyme and in the lysates were assayed in a reconstituted enzyme system as described previously (21) with some modifications. The assay mixture contained 0.1 nmol P450, 2 units NADPH-cytochrome P450 reductase, 40 μg L-dilauroylglyceryl-3-phosphocholine/ml, 0.015% 3-[(3-cholamidopropyl)dimethrylammonio]-1-propanesulfonate (CHAPS), 100 mM potassium phosphate, pH 7.4, 0.1% Emulgen 911, 1 mM EDTA, and 5 mM DTT in a final volume of 1 ml. Cholesterol was added to the reaction mixture in 20 μl Molecusol. The reaction was started by the addition of 1 mM NADPH and proceeded for 20 min at 37°C and stopped with 0.8% sodium cholate. 20α-Hydroxycholesterol was added as an internal standard. One unit of cholesterol oxidase was added to the reaction mixture and incubated for 10 min at 37°C. The reaction mixture was then extracted three times with 6 ml petroleum ether and combined extracts were then evaporated to dryness under nitrogen, dissolved in acetonitrile–methanol 70:30 (v/v) and analyzed on a C_{18} reversed-phase high performance liquid chromatography (HPLC) column (4.6 cm × 25 cm, 5 μm, Beckman Instruments, Fullerton, CA) as described previously (21). Chromatography was developed with acetonitrile–methanol 70:30 (v/v) at a flow rate of 0.8 ml/min and monitored at 240 nm. The oxidized products of 20α-hydroxycholesterol and 7α-hydroxycholesterol had retention times of approximately 8 and 11 min, respectively.

Other methods

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (22). SDS-PAGE-separated polypeptides were transferred electrophoretically onto an Immobilon-P membrane and incubated with antibody against rat liver cholesterol 7α-hydroxylase as previously described (5). The second antibody used was goat anti-rabbit IgG conjugated with alkaline phosphatase (1:3000 dilution, Bio-Rad). Color was developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (5). The cytochrome P450 concentration was determined by the reduced difference spectra of the CO-adduct according to Omura and Sato (23).

RESULTS

Expression of human cholesterol 7α-hydroxylase in *E. coli*

Previous work in our laboratory has resulted in the cloning of both the rat and human cholesterol 7α-hydroxylase cDNAs (4, 15) and also in the expression and purification of a catalytically active, truncated form of the rat cholesterol 7α-hydroxylase enzyme (16). In the present

TABLE 1. Subcellular distribution of human P450c7(Δ 2-24) in different buffers[a]

	Buffer I 100 mM potassium phosphate, pH 7.4, 0.5% sodium cholate	Buffer II 100 mM potassium phosphate, pH 7.4, 0.1% Triton X-100	Buffer III 100 mM potassium phosphate, pH 7.4	Buffer IV 10 mM potassium phosphate, pH 7.4
		%		
13,000 *g* Pellet	3.6	3.6	18.7	27.8
105,000 *g* Pellet	14.4	14.8	15.3	20.6
105,000 *g* Supernatant	82.0	81.6	66.0	51.6

[a] All buffers contained in addition 0.1 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF, and 20% glycerol. Values are the percentage of total P450 present in bacteria lysates suspended in each buffer.

investigation, a similar strategy was adopted to express the human cholesterol 7α-hydroxylase enzyme in *E. coli*. Initially, the human cDNA was inserted into the expression vector pKK233-2 (pKK/H7α1.5) and transformed into *E. coli* strain XLI-blue. No expression of enzyme activity or protein was observed by reconstitution of 7α-hydroxylase activity or immunoblot of *E. coli* lysates. pKK/H7α1.5 was then transformed into *E. coli* strains MV1304 and a panel of TOPP cells; none of these *E. coli* strains expressed human cholesterol 7α-hydroxylase. The human cDNA was then inserted into the pJL vector which was developed recently by Larson, Coon, and Porter (17). In addition to being a high copy number plasmid, the pJL vector possesses a translation enhancer sequence consisting of nine nucleotides preceding the conventional ribosomal binding site (Fig. 1, and refs. 24, 25). The pJL/H7α1.5 construct obtained was transformed into *E. coli* strains XLI-blue, MV1304, or a panel of six strains of TOPP cells. Only TOPP3 cells harboring pJL/H7α1.5 could express a functional human P450c7(Δ 2-24). Furthermore, a 100% increase in expression level and an improvement in the specific content of P450 in the purified protein were obtained when 0.2 mM δ-ALA was added to the TOPP3-pJL/H7α1.5 culture along with IPTG to a final concentration of 0.2 mM.

Expression of rat cholesterol 7α-hydroxylase in *E. coli*

To improve the level of expression of the previously developed rat P450c7(Δ 2-24) expression system, the truncated rat P450c7(Δ 2-24) cDNA was subcloned into the pJL vector. This construct (pJL/R7α1.5) was then transformed into *E. coli* strain MV1304. A fivefold increase in the expression level over that reported previously using pKK233-2 expression plasmid was obtained. In addition, a 100% increase in the specific content of P450 in the purified protein was obtained when the pJL vector was used for expression.

Subcellular distribution of human P450c7(Δ 2-24)

To determine the subcellular distribution and the solubility of expressed human P450c7(Δ 2-24) in *E. coli*, TOPP3 cells carrying pJL/H7α1.5 were lysed and suspended in different buffers with or without detergent (Table 1). When 100 mM potassium phosphate buffer was used to suspend cells in the presence of sodium cholate (buffer I) or Triton X-100 (buffer II), 15% of the P450 remained in the 105,000 *g* pellet and 82% were found in the supernatant in both buffers. When detergents were omitted in the lysis buffers, 15% of the P450 remained in the 105,000 *g* pellet and 66% were in the supernatant in 100 mM potassium phosphate buffer (buffer III). When 10 mM potassium phosphate buffer (buffer IV) was used to suspend cells, 21% of the P450 remained in the 105,000 *g* pellet and 51% were in the supernatant. These results indicate that 70-80% of P450 in the clear lysate are actually cytosolic when 10 mM or 100 mM potassium phosphate buffer was used in the absence of detergents.

Purification of the *E. coli*-expressed human P450c7(Δ 2-24) enzyme

Human cholesterol 7α-hydroxylase was purified as described under Experimental Procedures. The elution profile of the Octylamino-Sepharose 4B column is shown in **Fig. 2A.** The P450 fractions were detected in the second peak, and thus could be partially separated from other bacterial cytochromes (first peak). The P450 fractions eluted from this column revealed five to six bands

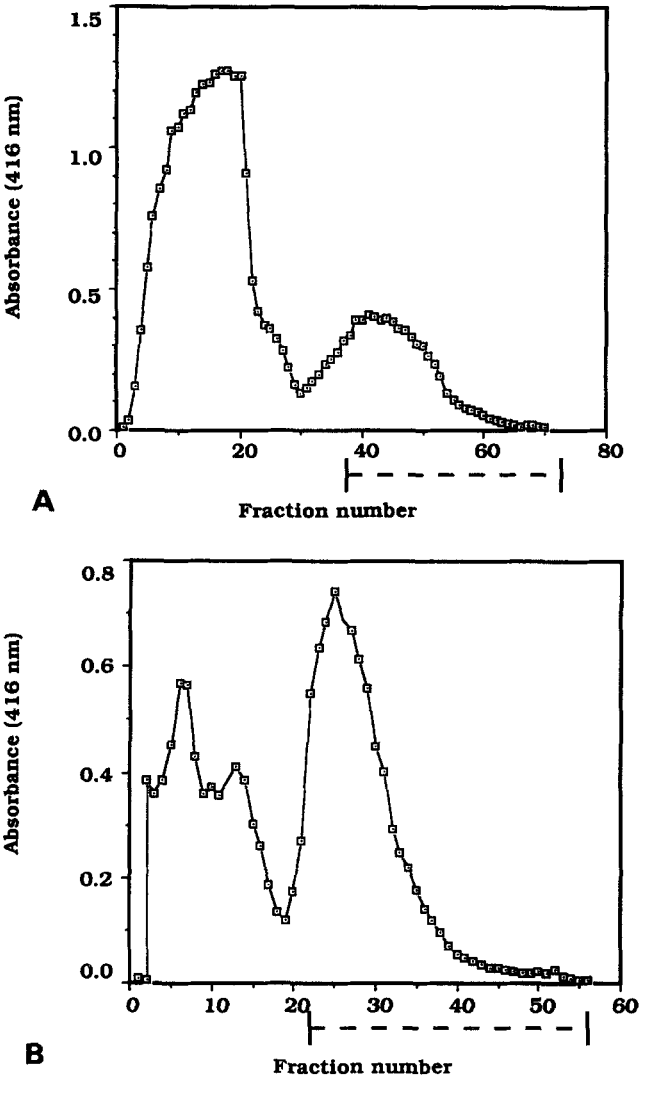

Fig. 2. Elution profiles of Octylamino-Sepharose 4B columns of bacteria expressed human and rat P450c7(Δ 2-24) enzymes. *E. coli* clear lysates were submitted to OAS-4B column chromatography. Both enzymes were applied to the columns and eluted in buffer A as described in Experimental Procedures. Five- and ten-ml fractions were collected from the rat and human enzyme preparations, respectively. Absorbance of column eluates was monitored at 416 nm. Fractions pooled for further purification by hydroxylapatite chromatography are indicated by a dashed line.

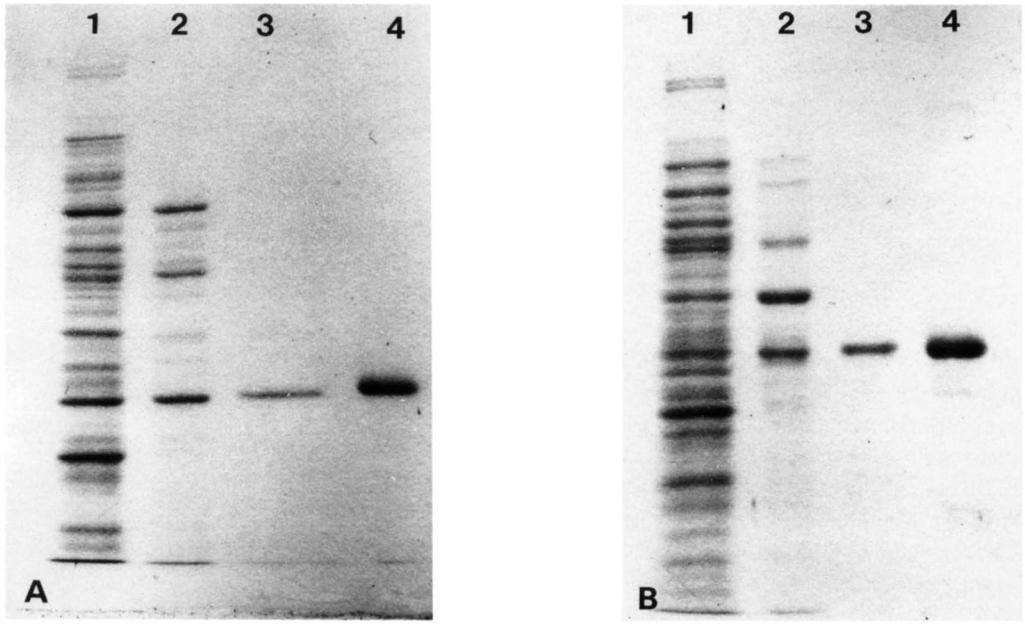

Fig. 3. SDS-polyacrylamide gel electrophoresis analysis of human and rat P450c7(Δ 2–24) eluted from column chromatographies. A: Human P450c7(Δ 2–24). B: Rat P450c7(Δ 2–24). Lane 1, total lysates of TOPP3-pJL/H7α1.5 and MV1304-pJL/R7α1.5, respectively, 15 μg each lane; lane 2, OAS-4B column eluates, 8 μg each lane; lane 3, eluates of human and rat enzyme preparations from the first hydroxylapatite columns, 2 μg and 3 μg each lane, respectively; lane 4, glutamate dehydrogenase molecular weight standard (53 kDa).

when analyzed on SDS-polyacrylamide gel electrophoresis (lane 2, **Fig. 3A**). These fractions were dialyzed and applied to a hydroxylapatite column. The human enzyme bound weakly to this column. When Emulgen 911 was eliminated from the wash and elution buffers, the recovery and purity of the human enzyme preparation were improved significantly. The purity of the human enzyme preparation eluted from this hydroxylapatite column was confirmed by SDS-polyacrylamide gel electrophoresis (lane 3, Fig. 3A). Only one band was detected by Coomassie blue staining of the gel and the electrophoretic mobility of the polypeptide was as expected. The reduced CO-difference spectrum of this purified enzyme is shown in **Fig. 4A.** The maximal absorption of the CO-complex

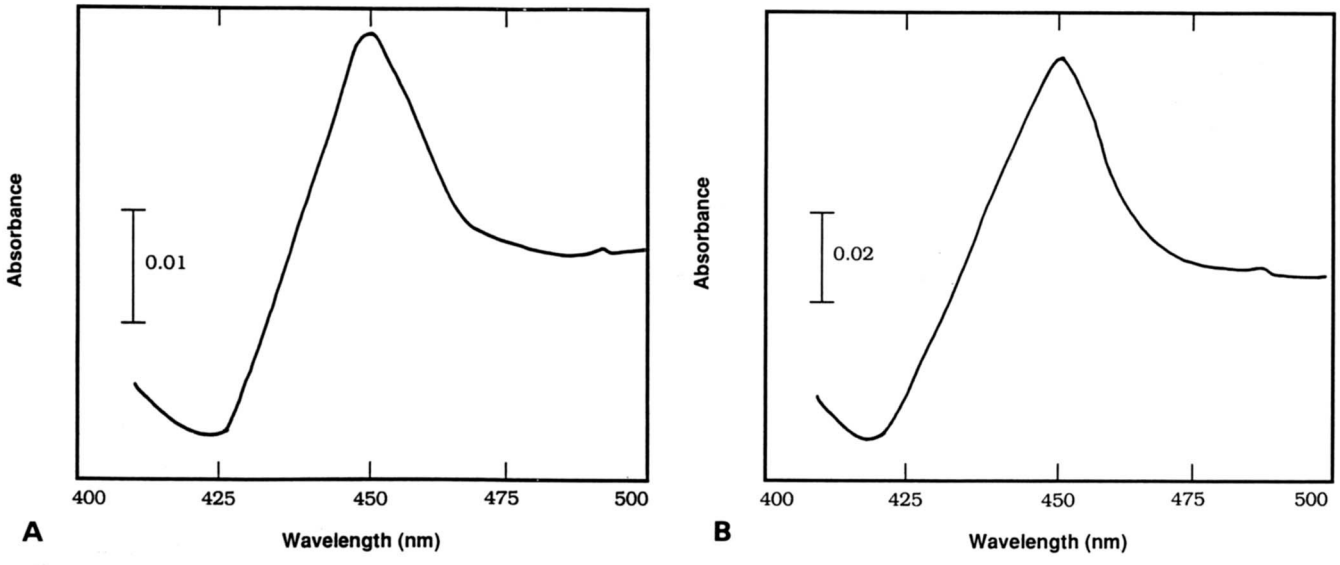

Fig. 4. The reduced difference spectra of CO-adduct of the purified human P450c7(Δ 2–24) (0.2 nmol/ml) (A) and rat P450c7(Δ 2–24) (0.5 nmol/ml) (B). Samples were dissolved in 100 mM potassium phosphate buffer, pH 7.4, 0.1% Emulgen 911, 0.1 mM EDTA, 0.1 mM DTT, and 20% glycerol. Spectra were determined as described in Experimental Procedures.

TABLE 2. Purification of the human P450c7(Δ 2-24) expressed in *E. coli*

Step	Total Protein mg	P450 Total P450 nmol	P450 Specific Content nmol/mg	Total Activity nmol 7α-hydroxycholesterol/ min	Specific Activity nmol 7α-hydroxycholesterol/ min/mg	Specific Activity nmol 7α-hydroxycholesterol/ min/nmol P450
Clear lysate	3045.00	152.00	0.05	40.00	0.01	0.26
Octylamino-Sepharose 4B	129.00	68.50	0.53	37.00	0.28	0.54
Hydroxylapatite-1	7.30	28.00	3.84	—	—	N.D.
Hydroxylapatite-2	6.50	25.00	3.85	31.20	4.80	1.25

N.D., not determined.

is at 450 nm and no 420 nm peak (denatured form) was detectable. A typical purification of the human enzyme is shown in **Table 2.** Purified enzyme (6.5 mg) was obtained from 8 liters of bacterial culture. The specific activity (nmol/min per mg) of the purified enzyme was increased 480-fold over the lysate. The yield of the purified enzyme based on the recovery of P450 was 16.2% and a 76-fold purification was achieved after the first hydroxylapatite column. The specific content of P450 in the purified preparation was 3.8 nmol/mg, which is higher than that of cholesterol 7α-hydroxylase purified from rat liver microsomes by us (5), but is substantially lower than that of rat enzyme reported by Ogishima, Deguchi, and Okuda (6) which had a specific content of about 9 nmol/mg of protein. It is known that cholesterol 7α-hydroxylase is a highly unstable P450 that may lose the heme prosthetic group during purification.

Purification of the *E. coli*-expressed rat P450c7(Δ 2-24) enzyme

The truncated rat cholesterol 7α-hydroxylase was purified as described under Experimental Procedures. The elution profile of the Octylamino-Sepharose 4B column is shown in Fig. 2B. The P450 fractions eluted from this column also showed five to six bands on SDS-polyacrylamide gel (lane 2, Fig. 3B). These fractions were further purified on a hydroxylapatite column and the purity of the P450 eluted was confirmed by a single, Coomassie blue-stained band on SDS-polyacrylamide gel (lane 3, Fig. 3B). Purification of the rat enzyme is shown in **Table 3.** Nine milligrams of purified enzyme was obtained from 4 liters of bacterial culture. The cholesterol 7α-hydroxylase specific activity (nmol/min per mg) was increased by 260-fold over the clear lysate. It should be mentioned that total activity in the clear lysate was apparently lower than that recovered in the Octyamino-Sepharose fractions due to the inhibitory effect of sodium cholate used for solubilization of cells. The yield of purified enzyme based on the recovery of P450 was 21% and a 35.5-fold purification was achieved. The specific content of P450 in the purified preparation was 4.6 nmol/mg, which is higher than the rat P450c7(Δ 2-24) purified previously (16). The reduced CO-difference spectrum of this purified rat P450c7(Δ 2-24) is shown in Fig. 4B. The maximal absorption of the CO-complex is at 450 nm and no P420 absorption was detectable.

Characterization of the purified P450c7(Δ 2-24)

Immunocrossreactivity of the purified rat and human P450c7(Δ 2-24) was studied by immunoblot. Rabbit polyclonal antibodies against rat microsomal cholesterol 7α-hydroxylase cross-reacted with the purified human enzyme (**Fig. 5**). An apparent difference in the electrophoretic mobility of these two polypeptides on SDS-polyacrylamide gel was consistently observed. The human P450c7(Δ 2-24) polypeptide has a faster electrophoretic mobility than the rat polypeptide, despite the fact that the molecular weights of *E. coli*-expressed human and rat P450c7(Δ 2-24) were the same.

Enzyme kinetics

The *E. coli*-expressed and truncated rat and human cholesterol 7α-hydroxylase were catalytically active as determined by a reconstitution assay. The K_m and V_{max} of

TABLE 3. Purification of the rat P450c7(Δ 2-24) expressed in *E. coli*

Step	Total Protein mg	P450 Total P450 nmol	P450 Specific Content nmol/mg	Total Activity nmol 7α-hydroxycholesterol/ *min*	Specific Activity nmol 7α-hydroxycholesterol/ *min/mg*	Specific Activity nmol 7α-hydroxycholesterol/ min/nmol P450
Clear lysate	1560.00	197.80	0.13	36.00	0.02	0.18
Octylamino-Sepharose 4B	72.00	164.80	2.28	143.00	1.98	0.86
Hydroxylapatite	9.10	42.00	4.61	48.00	5.26	1.14.

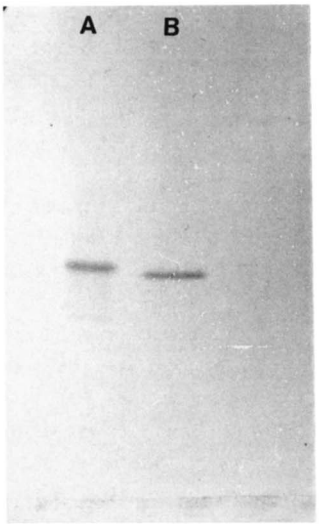

Fig. 5. Immunoblot of the purified, bacteria-expressed human and rat P450c7(Δ 2–24). Purified human and rat, bacteria-expressed P450c7(Δ 2–24) were separated by a 7.5% gel of SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto an Immobilon-P membrane, and incubated with polyclonal antibody against rat cholesterol 7α-hydroxylase; 0.5 μg of each protein was loaded in per lane. A: Rat P450c7(Δ 2–24). B: Human P450c7(Δ 2–24).

the purified P450c7(Δ 2–24) were determined by Lineweaver-Burk plots of the human or rat cholesterol 7α-hydroxylase activities measured with different concentrations of cholesterol (**Fig. 6**). The V_{max} and K_m were determined from these plots and turnover numbers were calculated from V_{max}. V_{max} and K_m for cholesterol, and turnover number of the human P450c7(Δ 2–24) were 0.13 nmol/min, 5.85 μM, and 1.3 min^{-1}, respectively, and those for the rat P450c7(Δ 2–24) were 0.14 nmol/min, 7.0 μM, and 1.5 min^{-1} respectively. The turnover numbers of the bacteria-expressed enzymes were much lower than 50 min^{-1} for the rat 7α-hydroxylase reported by Ogishima et al. (6), but were closer to those values reported previously by us (5, 16). The reason for such discrepancy is not known. The activities of the truncated enzymes are about 50% lower than the intact enzymes isolated from microsomes (5, 16). It is possible that the hydrophobic membrane-binding domain of 7α-hydroxylase may be involved in the interaction with P450 reductase and thus facilitated the electron transfer reaction. Molecusol, which was used in the assay of enzyme activity, efficiently solubilizes cholesterol and facilitates the delivery of cholesterol substrate to the active site, thus reducing the K_m for cholesterol without much effect on V_{max} or turnover number.

DISCUSSION

A detailed characterization of the human cholesterol 7α-hydroxylase has been hindered thus far by the extremely low level of this enzyme expressed in human liver and by the lack of an adequate supply of suitable human liver tissue for purification. Although two laboratories have reported the partial purification of human cholesterol 7α-hydroxylase (12, 13), these preparations were poorly characterized. Even the purification of cholesterol 7α-hydroxylase from rat livers was very difficult, laborious, and time-consuming (5).

We have previously succeeded in the expression and purification of a truncated rat P450c7(Δ 2–24) from *E. coli* harboring a cDNA lacking the first 24 amino acid codons of the N-terminal membrane-binding hydrophobic segment (16). However, expression of the human cholesterol 7α-hydroxylase in *E. coli* following a similar strategy and using the same vector (pKK233) and bacteria strain (XL1-blue) was unsuccessful, despite a high identity of nucleotide and amino acid sequence of 85% between the truncated rat and human cholesterol 7α-hydroxylase (15). Therefore, we screened several other *E. coli* strains transformed with two different human constructs pKK/H7α1.5 and pJL/H7α1.5. In our hands, the only expression system that expressed human cholesterol 7α-hydroxylase activity was the combination of pJL/H7α1.5 with a wild-type strain of *E. coli,* TOPP3 (26, 27). The combination of pKKH7α1.5 and TOPP3 was not successful in expressing the human enzyme and neither was pJL/H7α1.5 in XLI-blue. Although a functional enzyme could not be detected in other expression systems, we were able to detect 7α-hydroxylase mRNA in these *E. coli* lysates. It seems that combination of a suitable vector and a host is crucial for the expression of a func-

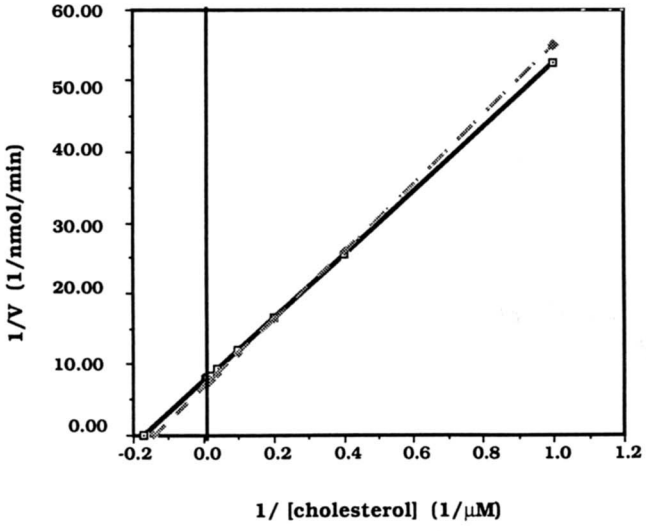

Fig. 6. Lineweaver-Burk plots of reconstitution assays of cholesterol 7α-hydroxylase activity. Human or rat P450c7(Δ 2–24), 0.1 nmol each were used per reconstitution assay as described in Experimental Procedures. Cholesterol was added in 20 μl of Molecusol. The correlation coefficients of the linear regression plots were 0.995 and 0.993 for the human (−⊡−) and rat (--■--) enzymes, respectively.

tional human cholesterol 7α-hydroxylase in *E. coli*. Although some factors such as the formation and stability of secondary structures near the ribosomal binding site of the vector (28, 29), the need for certain host heat shock proteins (30), and even the stability of the protein being expressed (31) have been specifically modified by investigators, a unique expression system still has to be developed for the expression of different proteins in *E. coli*.

Despite the fact that the amino acid sequence of the human and rat P450c7(Δ 2–24) exhibited 85% identity and that the molecular weights of these two proteins are similar, the behavior of these two expressed enzymes on SDS-polyacrylamide gel electrophoresis was different. Translation initiation from an internal methionine located at residue number 50 in the truncated human P450c7(Δ 2–24) could not account for this difference in electrophoretic mobility. It has been reported that P450 enzymes with deletions of more than 50 amino acids from their NH_2-terminus were unstable and inactive when expressed in *E. coli* (32, 33). It is also unlikely that this apparent difference in molecular weight is attributed to glycosylation of the expressed rat enzyme, as P450 enzymes are not known to be glycosylated.

Purified, bacteria-expressed P450c7(Δ 2–24) is most suitable for the determination of kinetic properties of cholesterol 7α-hydroxylase as bacteria do not contain any cholesterol. Although it has been reported that the enzyme isolated from rat liver was approximately 2.5-fold more active than the corresponding human enzyme (12), our data do not support this observation. We have determined that the truncated, *E. coli*-expressed human and rat P450c7(Δ 2–24) have similar K_m, V_{max}, and turnover number. These results provided strong evidence that rat and human cholesterol 7α-hydroxylase are essentially the same kinetically. We have also observed that the K_m values of the truncated rat and human P450c7(Δ 2–24) were lowered by 50% when Molecusol was used to solubilize cholesterol substrate instead of acetone or methanol. Molecusol has been found to improve the solubility of cholesterol, thus possibly making the substrate more available to the enzyme (34).

Our results indicated that most of the truncated human P450c7(Δ 2–24) enzymes expressed in *E. coli* were located in cytosol and the N-terminal hydrophobic membrane-anchor region was not required for catalytic activity. The unique structural feature of the truncated P450c7(Δ 2–24) is the highly charged N-terminus which may prevent the insertion of the N-terminus into the membrane. Truncated P4502E1 (17, 32), P4502B4 (32), P45021A1 (33), and P450c17 (35) have been expressed in *E. coli*. Most of these *E. coli*-expressed and truncated P450s were either membrane-bound or partially localized in the cytosol. The replacement of neutral amino acid residues by charged residues in positions 3 and 8 of P4502E1(Δ 3–29) increased the cytosolic localization of the truncated P4502E1 (32). The increase of N-terminus positively charged amino acid residues of the truncated P4502B4 also increased its cytosolic localization in *E. coli* (32). Two models of mammalian P450 topography in endoplasmic reticulum membrane have been proposed (36, 37). In the first model, a single N-terminus anchoring segment is inserted into the membrane and most of the P450 polypeptide is exposed to the cytosol with the N-terminal residues exposed to the lumen. In the second model, two hydrophobic segments (S1 and S2) in the N-terminus are separated by charged residues and form a hairpin loop which inserts into the membrane. In this model, the N-terminus is exposed to the cytosol (36). The N-terminal 29 amino acid residues of P4502C1 was demonstrated to be sufficient for anchoring soluble or secreted proteins to the endoplasmic reticulum membrane when this sequence was fused to these proteins (38). Our results and those findings mentioned above may support the single membrane insertion model, although other amino acid residues in the polypeptide chain may also interact with the membrane and contribute to the binding of mammalian P450s to endoplasmic reticulum membrane.

In conclusion, we have expressed a functional human cholesterol 7α-hydroxylase in *E. coli*. We have determined that the kinetic parameters for the *E. coli*-expressed, truncated human and rat P450c7(Δ 2–24) are essentially the same. This might imply that the extremely low level of cholesterol 7α-hydroxylase activity observed in the human liver could be attributed to a lower level of gene expression in the human liver and not to an actual difference in the kinetic properties of the rat and human cholesterol 7α-hydroxylase. This expression system could be used to produce large quantities of human cholesterol 7α-hydroxylase to study structure/function relationships by site-directed mutagenesis in the future.

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