Cholesterol 7α-hydroxylase from human liver: partial purification and reconstitution into defined phospholipid-cholesterol vesicles

Sandra K. Erickson and Bernhard Bösterling
Department of Medicine and Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305

Summary  Cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid synthesis, was shown to be copurified with human liver microsomal cytochrome P-450. When these cytochrome P-450 species were reconstituted in phospholipid-cholesterol vesicles together with NADPH-cytochrome P-450 reductase, high cholesterol 7α-hydroxylase activity was obtained in the presence of NADPH. The activity represented a twofold enrichment relative to activity was obtained in the presence of NADPH. The activity represented a twofold enrichment relative to total microsomal protein. Availability of such a preparation will allow further characterization of the enzyme and will also allow studies of its mechanisms of regulation.—Erickson, S. K., and B. Bösterling. Cholesterol 7α-hydroxylase from human liver microsomes. J. Lipid Res. 1981. 22: 872–876.

Supplementary key words  cytochrome P-450; bile acid synthesis

The rate of mammalian bile acid synthesis is believed to be controlled by the hepatic microsomal enzyme, cholesterol 7α-hydroxylase (1, 2). This enzyme is a NADPH-dependent monooxygenase system sensitive to carbon monoxide suggesting the participation of cytochrome P-450 (3–6). Bile acid synthesis in human liver homogenates was first described in 1968 by Björkhem et al. (7). As in the rat, cholesterol 7α-hydroxylase activity in humans appeared to be membrane-associated and appeared to be the rate-limiting enzyme for bile acid synthesis. The cholesterol 7α-hydroxylase activity in humans with various disorders of lipid metabolism was studied by Nicolaou et al. (8). The enzyme has never been purified. It has been partially purified from rabbit and rat livers (9, 10). However, recoveries of enzyme activity were low.

Because cholesterol 7α-hydroxylase is a multi-component system which includes cytochrome P-450, cytochrome P-450 reductase, and lipid (3–5), protein-protein, lipid-protein, and lipid-lipid interactions may all play a role in the regulation of this enzyme.

Materials and Methods

Materials
[4-14C]Cholesterol (50–60 mCi/mmol) and [1,2-3H]-cholesterol (40–60 mCi/mmol) were from New England Nuclear. NADPH was from Sigma. Servacel DEAE 23SS was from Accurate Chemical and Scientific Corporation. Biogel HTP was from BioRad. Cholic acid (sodium salt, A grade) was from Calbiochem. Triton N-101 was from Sigma. All other chemicals were reagent grade.

Livers
Livers were obtained within 1 hr after cessation of perfusion from heart donors in the Stanford Human Heart Transplantation Program. The protocol was approved by the Human Subjects Experimentation Committee.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.
Methods

Preparation of 7α-hydroxy-[1,2-3H]cholesterol. Labeled 7-ketocholesterol was prepared from [1,2-3H]cholesterol as described previously (13). It was reduced with NaBH₄ and the α and β epimers were separated by thin-layer chromatography (14). After separation, the radiochemical purity of the 7α epimer was assessed by thin-layer chromatography. More than 95% of the radioactivity comigrated with 7α-hydroxycholesterol in both the diethyl ether (14) and benzene–ether systems (13). No cholesterol or 7-ketocholesterol was detected by gas–liquid chromatography performed as described previously (13).

[^4C]Cholesterol. The radiolabeled cholesterol was diluted with unlabeled cholesterol which had been recrystallized sequentially from acetic acid, methanol, and acetone. The mixture was purified by thin-layer chromatography in diethyl ether. The sterol was scraped from the plate and eluted with chloroform.

Purification of human cytochrome P-450. Microsomes were prepared from human livers as previously described (12). The microsomes were resuspended in 0.15 M Tris/HCl, pH 7.8, containing 20% glycerol, 0.35% sodium cholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM N-α-p-tosyl-l-lysine chloromethyl ketone (TLCK), and 1 mM l-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) (protease inhibitor mix). The microsomes were washed at 4°C in a discontinuous sucrose gradient (12) containing the above inhibitor mix to remove glycogen and nonmembrane proteins. The purified microsomes containing 3.1 g protein were dissolved in 660 ml 10 mM Tris-HCl pH 7.8, containing 20% glycerol, 0.1 mM EDTA, 1.5% sodium cholate, and 1.5% Triton N-101. After stirring for 2 hr at room temperature, the solubilized microsomes were applied on a 5 × 15 cm DEAE cellulose column that had been equilibrated at 4°C with the same buffer. All further steps were at 4°C.

The eluate, which contained 65% of the total cytochrome P-450, was used unchanged for further purification on hydroxylapatite. After application of the cytochrome P-450 preparation, the Triton was removed by washing the 2.5 × 15-cm hydroxylapatite column with 1 liter of 30 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.3% sodium cholate. The column was then washed with 100 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.3% sodium cholate. The cytochrome P-450 was then eluted with 0.3 M potassium phosphate, pH 7.5, containing 20% glycerol, 0.4% sodium cholate, and 1 M sodium chloride. The preparation was dialyzed immediately against 0.3 M potassium phosphate, pH 7.5, containing 20% glycerol and 0.4% sodium cholate. After dialysis it was stored in liquid N₂.

Preparation of cytochrome P-450 reductase. Cytochrome P-450 reductase was isolated from rat liver microsomes after induction by phenobarbital, using a 2',5'-ADP Sepharose column (15). It reduced 40 μmoles of cytochrome c min⁻¹ mg protein⁻¹.

Reconstitution of microsomes and of cytochrome P-450 and cytochrome P-450 reductase into phospholipid vesicles with cholesterol. The same reconstitution technique was employed as was developed for the reconstitution of rabbit liver cytochrome P-450 into phospholipid vesicles (11). This preparation was shown by electron microscopy to yield a homogeneous population of non-aggregated vesicles (11).

Lipid:cholesterol vesicles. Human liver microsomal lipid was extracted with chloroform–methanol 2:1 under N₂. A trace amount of [^14C]cholesterol was added. After removal of the solvent under N₂, the lipid was dispersed in 5 ml of 0.3 M potassium phosphate buffer, pH 7.8, containing 20% glycerol. The mixture was dispersed by addition of 300 mg of cholate followed by sonication in a bath at 21°C for 2 min. The mixture dissolved completely within 3 hr at room temperature.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated from egg yolk (16). They were stored under N₂ at −20°C and remained colorless. They were mixed in chloroform–methanol 2:1 (v:v) at 64% PC, 32% PE, and 4% dipalmitoyl phosphatidic acid with labeled cholesterol added to give a mole ratio of 1:10 cholesterol:phospholipid. After complete removal of the solvent, 15 mg of this mixture was suspended in 5 ml of the same buffer as above containing 100 mg of sodium cholate and solubilized by 30-sec sonication in a bath at 21°C.

Human liver microsomes. Human liver microsomes were homogenized in 0.3 M potassium phosphate buffer, pH 7.8, containing 20% glycerol at a concentration of 22 mg protein per 5 ml. A trace amount of [^14C]cholesterol was mixed with egg phosphatidylcholine at a mole ratio of 1 and coated on the bottom of glass tubes by adding an aliquot of chloroform and drying under N₂. Microsomes were added and the cholesterol and microsomes were dispersed by sonication in a bath at 21°C for about 30 seconds. The microsomes and cholesterol were then dissolved by the addition of 140 mg sodium cholate.

Cytochrome P-450-cytochrome P-450 reductase. A solu-
tion of 5.1 μM purified human cytochrome P-450 and 1.7 μM purified rat cytochrome P-450 reductase in 0.3 M potassium phosphate buffer, pH 7.8, containing 20% glycerol, and 15 mg of the lipid mixture PC:PE:PA containing [14C]cholesterol (see above) in 5 ml of buffer was dispersed and 100 mg of cholate was added to dissolve the components.

Formation of vesicles. All solubilized samples were allowed to stand under N₂ for 2 hr at room temperature, followed by 5 hr at 4°C. All samples were then dialyzed under N₂ against 400 ml of 20 mM potassium phosphate buffer, pH 7.8, containing 20% glycerol for 4 days. The buffer which contained the PMSF-TPCK-TLCK protease inhibitor mix in the first ten volumes was changed twelve times. The resulting vesicle suspensions were then dialyzed against the same buffer at pH 7.4. On the fifth day after beginning the dialyses, the vesicles were characterized and assayed for enzyme activity.

Assay of the microsomal and vesicle preparations for cholesterol 7α-hydroxylase. All assays were incubated with stirring for 30 min at 25°C in the presence of oxygen (air) and 3 mM NADPH. The reaction was stopped by the addition of 25 ml of chloroform–methanol 2:1. 7α-Hydroxy[3H]cholesterol was then added as internal standard followed by 10 ml of chloroform–methanol–saturated acidic water (1 ml of H₂SO₄/liter). The phases were allowed to separate overnight at 4°C. The aqueous phase was removed by aspiration and the organic phase taken to dryness under N₂. The residue was taken up in a small volume of chloroform and the lipids were separated by thin-layer chromatography on silica gel H plates developed with diethyl ether. The bands corresponding to cholesterol were determined by counting an aliquot in toluene–Liquifluor and taking a second aliquot for assay by gas-liquid chromatography as described previously (12). Aliquots of the eluted 7α-hydroxycholesterol band were counted in toluene–Liquifluor for [14C] and for [3H]. Other aliquots were analyzed by thin-layer chromatography in the ether system and in ether–benzene 1:1. In both cases the [14C]/[3H] ratio remained constant within ±5%, suggesting that the [14C] label in this band was in 7α-hydroxycholesterol.

Chemical methods. Protein was determined by the biuret method (17) or according to Lowry et al. (18). Sterols were determined by gas–liquid chromatography as described previously (13). Radioactivities were determined by counting in toluene–Liquifluor (New England Nuclear) in a Beckman Model LS 150 liquid scintillation counter. All samples were corrected for spillover; [3H] spillover was 1% and [14C] spillover was 14%. Cytochromes P-450, P-420, and b₅ contents were determined as described by Imai and Sato (19).

RESULTS AND DISCUSSION

Human liver cytochrome P-450 was obtained in 26% overall yield. It had been purified 21-fold compared to the starting microsomes. The preparation contained 7.6 nmoles cytochrome P-450/mg protein. Less than 3% cytochrome P-420 was present. The preparation could be stored in liquid nitrogen after dialysis against 0.3 M potassium phosphate buffer, pH 7.5, containing 20% glycerol, for several months with no apparent loss in activity and no increased formation of cytochrome P-420. No cytochrome b₅ or NADPH cytochrome P-450 reductase activity was detectable in the preparations. The preparation consisted of a mixture of the major human hepatic cytochrome P-450 species (12).

In the reconstituted systems, 65% of the microsomal cytochrome P-450 was recovered in the reconstituted microsomes and 78% of the purified cytochrome P-450 in vesicles reconstituted with this preparation. No additional cytochrome P-420 was detected after reconstitution. In both preparations, the cytochrome P-450 to cytochrome P-450 reductase ratio was the same. All vesicle preparations were opalescent except for those from microsomes that were slightly turbid.

The monoxygenase system was reconstituted into vesicles containing phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and cholesterol because it was thought this would more closely approximate the membrane environment of the cholesterol 7α-hydroxylase in vivo. Thus, any results obtained with this system might more accurately mirror the in vivo situation.

In order to compare the microsomal and purified cytochrome P-450 activities more directly, the microsomes were subjected to the same reconstitution procedure as the cytochrome P-450:cytochrome P-450 reductase. Further, this procedure made it likely that all of the labeled cholesterol in the preparation was equally distributed in the membranes.

The rate of conversion of cholesterol to 7α-hydroxycholesterol by reconstituted vesicles is given in Table 1. In the microsomal system, 0.27 nmol 7α-hydroxycholesterol min⁻¹ nmol⁻¹ cytochrome P-450 or 0.10 nmol min⁻¹ mg⁻¹ protein was synthesized. In the purified cytochrome P-450-reconstituted system, 0.56 nmol of 7α-hydroxycholesterol min⁻¹ nmol⁻¹ cytochrome P-450 or 4.3 nmol min⁻¹ mg⁻¹
protein was formed. This represents a 43-fold purification over the original microsomes. The values were corrected for nonenzymatic conversion of cholesterol to 7α-hydroxycholesterol by determining the recovery of radioactivity as 7α-hydroxycholesterol in lipid vesicles containing no protein.

Some properties of the microsomal system were studied in more detail. The dependence of cholesterol 7α-hydroxylation on cytochrome P-450 (or microsomal protein) and time was investigated (Fig. 1A and B). The reaction was linear up to about 0.75 nmol cytochrome P-450 and up to about 60 min.

Addition of 1 mM dithiothreitol to the assays resulted in enhanced activity (up to 285%) as has been reported for the rat liver enzyme (5, 6). This suggests that reduced sulfhydryl groups may play an important role in the hydroxylation. Inclusion of phenyl-N-t-butyl nitrotrate (18 mg/ml), a free-radical trapping agent, in the assay mixtures had no effect or slightly enhanced the activity suggesting that cholesterol 7α-hydroxylation mediated by the P-450 system does not proceed via a free radical mechanism.

It is difficult to compare the activities of microsomal cholesterol 7α-hydroxylase obtained in this study with those reported previously for human liver (7, 8). If one assumes that 7α-hydroxycholesterol production is rate-limiting for the overall rate of bile acid synthesis (1, 2, 7), the daily estimated rate of bile acid synthesis based on the value reported here for microsomes of 0.10 nmol 7α-hydroxycholesterol produced min⁻¹·mg⁻¹ microsomal protein is about 1 g per day for a liver weight of 1.5 kg of which 15 g is assumed to be endoplasmic reticulum. The bile acid synthesis rate in humans is estimated as about 0.6 g per day (20-22), with a range of 265-875 mg reported for ten patients (20). The higher value calculated here may represent partial derepression of the 7α-hydroxylase activity in our patients. Thus, the method for analysis of microsomal cholesterol 7α-hydroxylase activity in human liver described in this report appears to result in values that may be close to those estimated in vivo.

The purification of cytochrome P-450 from human liver, its reconstitution into defined lipid vesicles, and the demonstration of enriched cholesterol 7α-hydroxylase activity in these preparations make possible detailed studies of the regulation of this enzyme. Suggestions that the enzyme is regulated by membrane fluidity and by substrate availability may now be amenable to investigation as well as studies on the role of cytosolic factors in expression of cholesterol 7α-hydroxylase activity.

### Table 1. Cholesterol 7α-hydroxylase activity in microsomes from human liver and in a reconstituted cytochrome P-450 preparation

<table>
<thead>
<tr>
<th>Lipid Preparation</th>
<th>7α-Hydroxycholesterol (nmol)</th>
<th>Specific Activity (nmol 7α-Hydroxycholesterol/nmol cytochrome P-450·min⁻¹·mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal lipid vesicles</td>
<td>108</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>Microsomes</td>
<td>154 ± 26</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>PC:PE:PA vesicles</td>
<td>112</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>Cyt P-450-cyt P-450 reductase vesicles</td>
<td>357 ± 41</td>
<td>0.56 ± 0.06</td>
</tr>
</tbody>
</table>

*The values are corrected for 7α-hydroxycholesterol production due to nonenzymatic oxidation.*

Microsomes were prepared, cytochrome P-450 and cytochrome P-450 reductase purified, and vesicles were prepared as described in Methods. Each assay contained 5.7 nmol of cytochrome P-450 as microsomes or 14.4 nmol of cytochrome P-450 as the reconstituted cytochrome P-450 monoxygenase system in vesicles. Control assays contained lipid vesicles alone. The buffer contained 0.3 M potassium phosphate, pH 7.8, and 20% glycerol. Incubation was for 30 min; the gas phase was air; the temperature was 25°C. The reactions were initiated by addition of NADPH and terminated with chloroform: methanol 2:1. 7α-Hydroxy[3H]cholesterol was then added as internal standard and the lipids were separated as described in Methods. The results are the average of determinations from two different livers.
REFERENCES


