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

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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 cytochrome P-450 and 43-fold 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: partial purification and reconstitution into defined phospholipid-cholesterol vesicles. *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 Nicolau 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 multicomponent 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. Cholesterol, the substrate for the reaction, is also a membrane component. Thus, the sterol itself can have a regulatory role both as substrate and as a component of the membrane in which the enzyme is located. As a first step in understanding how the human enzyme might be regulated, it was desirable to have purified preparations of the components that could be reconstituted into defined phospholipid vesicles containing cholesterol.

Bösterling et al. (11) showed recently that purified cytochrome P-450 and cytochrome P-450 reductase from rabbit liver can be incorporated into a phospholipid membrane consisting of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE). The resulting reconstituted preparations had structural and functional similarities to the endoplasmic reticulum. Additionally, higher enzyme activities and lower production of peroxides were observed than if dilauryl phosphatidylcholine, commonly used to reconstitute P-450 systems, was utilized. Thus, this reconstitution system may prove superior for studying cholesterol 7α-hydroxylase. In addition, cytochrome P-450 has been partially purified recently from human liver by Bösterling and Trudell (12). When reconstituted into PC-PE vesicles as above, the preparation metabolized halothane.[1] We have used a similar preparation to determine whether such a system also showed cholesterol 7α-hydroxylase activity. We wish to report that the enzyme activity is indeed present and that it has been purified 43-fold relative to the microsomal activity.

MATERIALS AND METHODS

Materials

[4-^{14}C]Cholesterol (50–60 mCi/mmol) and [1,2-^{3}H]-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-1-phenylethylchloromethyl ketone.

[1] Bösterling, B. Unpublished observations.

Methods

Preparation of 7α-hydroxy-[1,2-^{3}H]cholesterol. Labeled 7-ketocholesterol was prepared from [1,2-^{3}H]cholesterol as described previously (13). It was reduced with $NaBH_4$ 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).

[^{14}C]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. More than 95% of the radioactivity comigrated with cholesterol as judged by thin-layer chromatography in benzene–ether 1:1 and in diethyl ether. No detectable radioactivity comigrated with 7α-hydroxycholesterol in these systems.

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 N_2.

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* $min^{-1} \cdot mg\ protein^{-1}$.

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 N_2. A trace amount of [^{14}C]cholesterol was added. After removal of the solvent under N_2, 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 N_2 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 [^{14}C]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 N_2. 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 [^{14}C]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 N_2 for 2 hr at room temperature, followed by 5 hr at 4°C. All samples were then dialyzed under N_2 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-Hydroxyl[^{3}H]cholesterol was then added as internal standard followed by 10 ml of chloroform–methanol–saturated acidic water (1 ml of H_2SO_4/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 N_2. 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 and to 7-α-hydroxycholesterol were scraped from the plates and eluted with chloroform. The samples were taken to dryness under N_2 and the residues were dissolved in ethanol. The specific activity of the cholesterol was 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 ^{14}C and for ^{3}H. Other aliquots were analyzed by thin-layer chromatography in the ether system and in ether–benzene 1:1. In both cases the ^{14}C/^{3}H ratio remained constant within ±5%, suggesting that the ^{14}C 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; ^{3}H spillover was 1% and ^{14}C spillover was 14%. Cytochromes P-450, P-420, and b_5 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 b_5 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 monooxygenase 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 $min^{-1} \cdot nmol^{-1}$ cytochrome P-450 or 0.10 nmol $min^{-1} \cdot mg^{-1}$ protein was synthesized. In the purified cytochrome P-450-reconstituted system, 0.56 nmol of 7α-hydroxycholesterol $min^{-1} \cdot nmol^{-1}$ cytochrome P-450 or 4.3 nmol $min^{-1} \cdot mg^{-1}$

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 μmol 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 nitrone (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 $min^{-1} \cdot mg^{-1}$ 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.

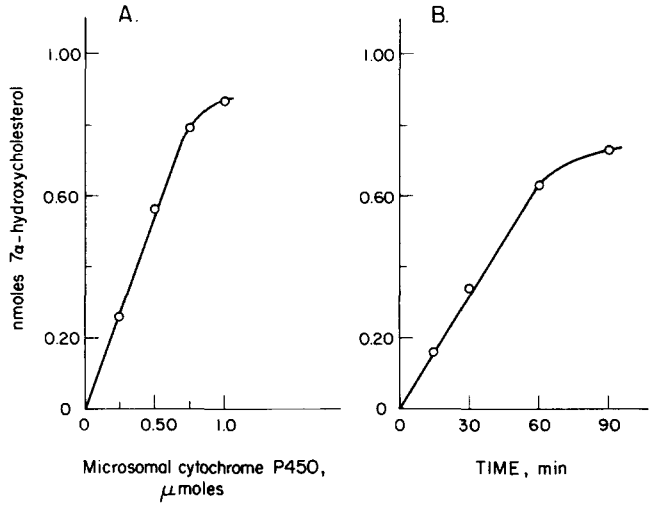

Fig. 1. Dependence of human liver microsomal cholesterol 7α-hydroxylase activity on cytochrome P-450 concentration (A) and time (B). Liver microsomes were prepared and [^{14}C]cholesterol-containing vesicles prepared as described in the text. All values were corrected for nonenzymatic lipid oxidation by subtracting values obtained for microsomal lipid vesicles incubated under exactly the same conditions. The incubations were performed at 25°C and started by the addition of NADPH. The reactions were terminated by the addition of chloroform–methanol 2:1 and the lipid was extracted. 7α-Hydroxy[^{3}H]cholesterol was added as internal standard and the products were separated by thin-layer chromatography as described in the text. The assay time in A was 30 min. The assays in B contained 0.5 μmol cytochrome P-450. Note: The activity measured in this liver was low compared to that in livers from three other individuals. Interestingly, the phospholipid:cholesterol mole ratio was 30, or about twice as high as that in the other individuals.

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

	nmol 7α-Hydroxycholesterol in 30 min	Specific Activity nmol 7α-Hydroxycholesterol $nmol^{-1}$ cyt P-450 $\cdot min^{-1}$ [a]
Microsomal lipid vesicles	108	
Microsomes	154 ± 26	0.27 ± 0.06
PC:PE:PA vesicles	112	
Cyt P-450-cyt P-450 reductase vesicles	357 ± 41	0.56 ± 0.06

[a] 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 monooxygenase 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[^{3}H]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.

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