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7α-Hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid by cytochrome P-450 in pig liver microsomes

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Pig liver microsomes were found to catalyze the 7α-hydroxylation of serveral potential bile acid precursors besides cholesterol. 26-Hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid were all efficiently converted into the 7α-hydroxylated products. Two cytochrome *P*-450 fractions showing 7α-hydroxylase activity could be isolated. One fraction catalyzed 7α-hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid but was inactive towards cholesterol. The other fraction catalyzed 7α-hydroxylation of cholesterol in addition to the other substrates. 26-Hydroxycholesterol in equimolar concentration did not inhibit the cholesterol 7α-hydroxylase activity of this fraction. It is concluded that liver microsomes contain a cytochrome *P*-450 catalyzing 7α-hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid. The results indicate that this cytochrome *P*-450 is different from that catalyzing 7α-hydroxylation of cholesterol.

Cytochrome *P*-450; 7α-Hydroxylase; 26-Hydroxy-cholesterol; 3β-Hydroxy-5-cholestenoic acid; 3β-Hydroxy-5-cholenoic acid

1. INTRODUCTION

The biosynthesis of primary bile acids involves introduction of a 7α-hydroxyl group into the steroid nucleus. Generally, the 7α-hydroxylation of cholesterol is regarded as the initial and rate-limiting step. Alternative pathways not involving 7α-hydroxylation of cholesterol have been described [1–3]. Administration of 26-hydroxycholesterol to rat, hamster, rabbit and man has resulted in the formation of 7α-hydroxylated products [3–5]. Recently, Axelson and Sjövall [6] proposed a model for the biosynthesis of bile acids in humans including 7α-hydroxylation of 26-oxygenated intermediates. Whereas the microsomal cytochrome *P*-450 catalyzing 7α-hydroxylation of cholesterol in rat liver has been well characterized [7–9] there is no report on microsomal 7α-hydroxylation of other potential bile acid precursors such as 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid or 3β-hydroxy-5-cholenoic acid. In fact, apparently homogeneous cholesterol 7α-hydroxylase purified from rat liver microsomes was found to be inactive toward 3β- hydroxy-5-cholenoic acid [7]. However, species differences may exist and the rat may not be a representative animal for evaluation of pathways of bile acid biosynthesis in man [6]. In contrast to rat, the pig has a pattern of neutral and acidic C_{27}-steroids in plasma similar to that in man (unpublished data), possibly reflecting similarities in pathways of bile acid biosynthesis. Therefore, the 7α-hydroxylation of potential bile acid precursors with a 3β-hydroxy-5-ene structure was studied in fractions of pig liver. The present communication demonstrates the presence of cytochrome *P*-450 in pig liver microsomes catalyzing 7α-hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid.

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2. EXPERIMENTAL

2.1. *Chemicals*

DEAE-Sephorose and S-Sepharose were from Pharmacia. Other chemicals, reagents and column packing materials were those used previously in our laboratories [10,11]. Unlabeled and $^{2}H_{7}$-labeled 26-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid and their 7α-hydroxylated analogues were all synthesized from kryptogenin (a kind gift from Dr. L. Tökes, Syntex, Palo Alto, CA, USA) Unlabeled 3β-hydroxy-5-cholenoic acid was from Steraloids Inc. (Wilton, NH, USA) and the $^{2}H_{5}$-labeled version was a kind gift from Prof. M. Tohma, Hokkaido, Japan. The corresponding 7α-hydroxylated compounds were synthesized. The synthesis of all the compounds will be reported separately. The unlabeled and labeled steroids were used as substrates for the incubations and as internal standards for the analyses, respectively.

2.2. *Purification of 7α-hydroxylation enzymes*

Liver microsomes were prepared from castrated, otherwise untreated, 6-month-old male pigs. The microsomes were suspended in 0.1 M Tris-HCl buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol, adjusted to a concentration of 6 mg protein per ml, solubilized with 1.8% (w/v) sodium cholate and subjected to poly-

ethylene glycol precipitation as described previously [10]. The proteins precipitating between 7–14% polyethylene glycol were collected by centrifugation, homogenized and dissolved in a 0.01 M phosphate buffer, pH 7.6, containing 20% (v/v) glycerol, 0.4% (w/v) Emulgen 911, 0.1 mM EDTA and 0.1 mM dithiothreitol. All buffers used in the purification procedure contained 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol. Phosphate buffer was used as the potassium salt. The solution was subjected to chromatography on DEAE-Sepharose (7.5 × 30 cm), equilibrated in 0.01 M phosphate buffer, pH 7.6, containing 0.4% Emulgen 911. Cytochrome *P*-450 was eluted with the equilibrating buffer. The fractions active in 7α-hydroxylation towards cholesterol and 26-hydroxycholesterol were pooled and applied to a hydroxylapatite column (6 × 30 cm) equilibrated in 0.01 M phosphate buffer, pH 7.4, containing 0.2% Emulgen 911. The column was washed with the equilibrating buffer and with a 0.1 M phosphate buffer, pH 7.4, containing 0.2 % Emulgen 911. Cytochrome *P*-450 active in 7α-hydroxylation towards cholesterol and 26-hydroxycholesterol eluted with a 0.2 M phosphate buffer, pH 7.4, containing 0.2% Emulgen 911. The active fractions were concentrated to 10 ml using an ultrafiltrating unit (Diaflo) with a PM30 filter. The concentrated sample was dialyzed overnight against 0.01 M phosphate buffer, pH 7.8, containing 0.4% Emulgen 911 and subjected to High Performance Liquid Chromatography (HPLC) using an S-Sepharose column (2.6 × 30 cm) equilibrated in the same buffer. The flow rate was 4.0 ml/min and cytochrome *P*-450 was eluted in a stepwise fashion with increasing concentrations of phosphate in the equlibrating buffer. After washing the column with equilibrating buffer, 2 peaks (I and II) containing cytochrome *P*-450 were eluted with 0.05 M phosphate and 0.2 M phosphate, respectively. The cytochrome *P*-450 fractions showed several protein bands upon SDS-polyacrylamide gel electrophoresis and contained 2.1 and 1.5 nmol of cytochrome *P*-450/mg of protein. The purification procedure is summarized in Fig 1.

Protein was determined by the method of Bradford [12]. Cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase activity were determined as described earlier [13,14].

2.3. *Incubation procedure*

Incubations were carried out at 37°C for 30 min with liver microsomes and for 10 min with reconstituted systems. The substrates [^{14}C] cholesterol and unlabeled 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid, 10 μg dissolved in 25 μl of aceton, were incubated with microsomes (1 nmol of cytochrome *P*-450) or cytochrome *P*-450 fractions (0.1 nmol), 5 mM dithiothreitol and 1 μmol of NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. In incubations with partially purified cytochrome *P*-450 fractions, 2 U of microsomal NADPH-cytochrome *P*-450 reductase were added. To incubations with [^{14}C]cholesterol, Triton X-100 was added at a concentration of 0.05% (w/v) to facilitate substrate dissolution.

Incubations with [^{14}C]cholesterol were terminated with 5 ml trichloroethane/methanol (2:1) and analyzed on the same day as described previously [10]. Incubations with the other substrates were terminated with 3 ml of methanol, mixed and stored at -20°C until analyzed.

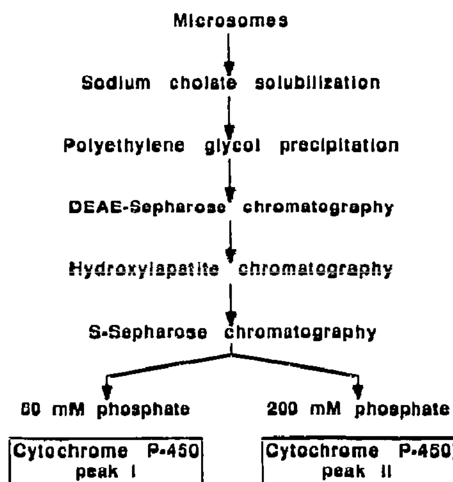

Fig. 1. Schematic procedure for the separation of 7α-hydroxylating cytochrome *P*-450 species from pig liver microsomes.

2.4. *Gas chromatographic-mass spectrometric analyses*

The methanolic incubation mixture was centrifuged and to the supernatant was added ^{2}H-labeled 7α-hydroxysteroids as internal standards. Hydroxysteroids and bile acids were then isolated in principle as described previously for these compounds in cultured cells [15]. Briefly, the sample (in 75% aqueous methanol) was passed through a column of octadecylsilane-bonded (ODS) silica. The effluent was either diluted with water to reach a concentration of 40% aqueous methanol (for isolation of hydroxysteroids) or concentrated to a water solution (for bile acids) and was then re-extracted on the same column. Steroids more polar than cholesterol were then eluted with 85% aqueous methanol. Following dilution to 50% aqueous methanol the hydroxysteroids were re-extracted on a second column of ODS-bonded silica from which they were eluted with methanol. Emulgen 911 was removed by chromatography on a column (8.5 × 0.4 cm) of Lipidex 1000 in 50% aqueous methanol. Following a wash of the column with 10 ml of 50% aqueous methanol and 5 ml of 60% aqueous methanol, hydroxysteroids were eluted with 10 ml of methanol and converted into trimethylsilyl ethers. Bile acids were isolated from the 85% methanolic eluate by chromatography in an anion exchanger, TEAP-LH-20, and converted into methyl ester trimethylsilyl ether derivatives.

The steroid metabolites were analyzed by gas chromatography-mass spectrometry as previously described [11,15]. The identification of a steroid was based on the retention time and the complete mass spectrum which were compared with those of the authentic steroid. Quantitation was achieved by comparing peak areas in fragment ion current chromatograms given by the steroid metabolites with those given by the corresponding ^{2}H-labeled internal standards.

3. RESULTS AND DISCUSSION

Pig liver microsomes were found to catalyze the 7α-hydroxylation of several potential bile acid precursors in addition to cholesterol. Table I summarizes the

Table I

7α-Hydroxylase activities in microsomes and partially purified cytochrome *P*-450 fractions from pig liver

Fraction	7α-Hydroxylase activity (mol/min · mol *P*-450)			
	Cholesterol	26-hydroxycholesterol	3β-hydroxy-5-cholestenoic acid	3β-hydroxy-5-cholenoic acid
Microsomes	0.001	0.06	0.02	0.05
S-Sepharose peak I	1.20	0.89	0.06	0.38
S-Sepharose peak II	≤0.02	1.22	0.15	0.31

Incubations were carried out as described in Experimental. When NADPH or NADPH-cytochrome *P*-450 reductase was omitted the 7α-hydroxylase activity towards 26-hydroxycholesterol was less than 1% compared to that with complete system. The corresponding values for 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid were 30% and 20%, respectively.

results from incubations with microsomes, NADPH and unlabeled 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid, 3β-hydroxy-5-cholenoic acid or ^{14}C-labeled cholesterol. The most efficient 7α-hydroxylation was with 26-hydroxycholesterol followed by 3β-hydroxy-5-cholenoic acid, 3β-hydroxy-5-cholestenoic acid and cholesterol. The rate of hydroxylation of endogenous and unlabeled cholesterol was also analyzed and found to be essentially the same as with the labeled cholesterol. 7α-Hydroxylation due to auto-oxidation seems to be excluded since the 7β-isomer accounted for less than 5% of the products formed from 26-hydroxycholesterol. Blank incubations with boiled microsomes did not yield detectable amounts of 7α-hydroxylated products.

The cytochrome *P*-450 in pig liver microsomes catalyzing 7α-hydroxylation of cholesterol had previously been solubilized and partially purified [16]. In order to study the nature of the 7α-hydroxylase active towards 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid, microsomal protein was solubilized and subjected to chromatography (Fig. 1). The 7α-hydroxylase activities towards cholesterol and 26-hydroxycholesterol were monitored during the purification procedure. In the first steps the 7α-hydroxylase activities co-purified. However, HPLC on S-Sepharose yielded 2 peaks of 7α-hydroxylating cytochrome *P*-450 with different substrate specificities. The cytochrome *P*-450 in peak I was active towards both cholesterol and 26-hydroxycholesterol. The cytochrome *P*-450 in peak II showed no or only very low activity towards cholesterol but catalyzed an efficient 7α-hydroxylation of 26-hydroxycholesterol (Table I). This reaction required NADPH-cytochrome *P*-450 reductase and NADPH, clearly demonstrating that the enzyme is a member of the cytochrome *P*-450 superfamily. The possibility of lack of 7α-hydroxylase activity towards the ^{14}C-labeled cholesterol due to dilution with endogenous cholesterol can be excluded since less than 2 μg/incubation of unlabeled cholesterol was found in peak II. The fractions eluted between the 2 peaks showed very low activity towards both substrates. As also shown in Table I, the cytochrome *P*-450s, in both peaks were active towards 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid.

Since cholesterol 7α-hydroxylase in peak I from the S-Sepharose chromatography could not be obtained free from activity towards 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid it cannot be excluded that this cytochrome *P*-450 is able to catalyze 7α-hydroxylation of other substrates than cholesterol. However, the results of inhibition experiments indicate that the cholesterol 7α-hydroxylase is not active towards 26-hydroxycholesterol. Thus, the cholesterol 7α-hydroxylase activity in the cytochrome *P*-450 fraction was not inhibited by an equimolar amount of 26-hydroxycholesterol (Table II).

Table II

Effect of 26-hydroxycholesterol on 7α-hydroxylation of cholesterol by cytochrome *P*-450 peak I

	7α-hydroxylation of cholesterol (mol/min·mol *P*-450)
Control	1.40
+10 μM 26-hydroxycholesterol	1.43
+20 μM 26-hydroxycholesterol	1.54
+25 μM 26-hydroxycholesterol	1.33

Incubations were carried out as described in Experimental except that 10 μM [^{14}C]cholesterol was used as substrate and various amounts of 26-hydroxycholesterol were added.

Conversely, cholesterol did not inhibit 7α-hydroxylation of 26-hydroxycholesterol in equimolar concentration. 26-Hydroxycholesterol was used in these inhibition experiments since this compound is physically and structurally more similar to cholesterol than the C_{27}- and C_{24}-acids. The results from the inhibition experiments together with the fact that it was possible to isolate a cytochrome *P*-450 fraction active towards 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid but essentially inactive towards cholesterol strongly indicate that the cholesterol 7α-hydroxylase is not involved in hydroxylation of side chain oxygenated substrates. This contention is supported by a report showing that apparently homogeneous rat liver cholesterol 7α-hydroxylase was not active towards 3β-hydroxy-5-cholenoic acid [7].

In conclusion, the present communication demonstrates cytochrome *P*-450-dependent 7α-hydroxylation of 26-hydroxycholesterol, 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid in liver microsomes. The results strongly indicate the presence of multiple microsomal sterol 7α-hydroxylases and provide a basis for their further purification and studies on their structures, regulation and roles in bile acid biosynthesis.

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REFERENCES

[1] Björkhem, I. (1985) in: Sterols and Bile Acids (Danielsson, H. and Sjövall, J. eds) pp. 231–278, Elsevier, Amsterdam.
[2] Mitropoulos, K.A., Avery, M.D., Myant, N.B. and Gibbons, G.F. (1971) Biochem. J. 130, 363–371.
[3] Javitt, N.B., Kok, E., Carubbi, F., Blizzard, T., Gut, M. and Byon, C.Y. (1986) J. Biol. Chem. 261, 12486–12489.
[4] Ayaki, Y., Kok, E. and Javitt, N.B. (1989) J. Biol. Chem. 264, 3818–3821.
[5] Anderson, K.E., Kok, E. and Javitt, N.B. (1972) J. Clin. Invest. 51, 112–117.
[6] Axelson, M. and Sjövall, J. (1990) J. Steroid Biochem. 36, 631–640.

[7] Ogishima, T., Deguchi, S. and Okuda, K. (1987) J. Biol. Chem. 262, 7646–7650.
[8] Chiang, J.Y.L., Miller, W.F. and Lin, G.M. (1990) J. Biol. Chem. 265, 3889–3897.
[9] Jelinek, D.F., Andersson, S., Slaughter, C.A. and Russel, D.W. (1990) J. Biol. Chem. 265, 8190-8197.
[10] Andersson, S., Boström, H., Danielsson, H. and Wikvall, K. (1985) Methods Enzymol. 111, 364–377.
[11] Axelson, M., Mörk, B. and Sjövall, J. (1988) J. Lipid Res. 29, 629–641.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[14] Masters, B.S.S., Williams Jr., C.H. and Kamin, H. (1967) Methods Enzymol. 10, 565–573.
[15] Axelson, M. Mörk, B. and Everson, G.T. (1991) J. Biol. Chem. 266 (in press).
[16] Boström, H. (1986) J. Lipid Res. 27, 807–812.